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EXPRESSION OF HAEM OXYGENASE-1 AND INDUCIBLE NITRIC OXIDE SYNTHASE IN HORSES WITH LOWER AIRWAY INFLAMMATION

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©

ABSTRACT

The heat shock protein haem oxygenase-1 (HO-1) and the enzyme inducible nitric oxide synthase (iNOS) are both induced during conditions of oxidative stress and by pro-inflammatory mediators. Both HO-1 and iNOS may therefore be induced by a range of disease states that result in tissue inflammation. Both have been demonstrated to have potentially beneficial anti-inflammatory effects. Both HO-1 and iNOS and their respective gaseous products carbon monoxide and nitric oxide have being the subject of investigation as potential therapeutic targets for human respiratory disease, most notably asthma. The pathogenesis of inflammatory diseases of the equine lower respiratory tract has many similarities with human asthma and both HO-1 and iNOS have been demonstrated in fixed equine pulmonary tissue.

The aim of the study was to investigate the expression of HO-1 and iNOS in the equine respiratory tract. The primary objective was to investigate whether HO-1 and iNOS were expressed within leukocytes from equine BALF and to semi-quantify expression in different cell types. If it proved possible to characterise and quantify expression, additional aims were to investigate associations between HO-1 expression, iNOS expression, clinical signs of respiratory disease and markers of oxidative stress in exhaled breath condensate.

We were successful in demonstrating the expression of both HO-1 and iNOS in leukocytes from equine BALF. Expression was most intense in macrophages and neutrophils which was consistent with previous studies performed in other species. We did not identify an association between the degree of HO-1 or iNOS staining in leukocytes from BALF and the severity of clinical respiratory disease. We also failed to identify an association between expression of HO-1 and iNOS in leukocytes from BALF and markers of oxidative stress in exhaled breath condensate. Further investigations ought to be performed into the expression and activity of both HO-1 and iNOS in the equine respiratory tract.

AUTHOR'S DECLARATION

I, David Rendle, declare that the work in this thesis is original and was carried out solely by myself or with due acknowledgments. It has not been submitted in any form for another degree of professional qualification.

Part of this thesis has been submitted for publication elsewhere:

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LIST OF ABBREVIATIONS

AHR	airway hyperreactivity
AP-1	transcription factor AP-1
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
bpm	breaths per minute
cGMP	cyclic guanosine monophosphate
cNOS	cyclic nitric oxide synthase
°C	degrees celcius
CO	carbon monoxide
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
DNA	deoxyribose nucleic acid
EBC	exhaled breath condensate
EDRF	endothelium derived relaxating factor
EIPH	exercise induced pulmonary haemorrhage
ELF	epithelial lining fluid
eNANC	non-adrenergic, non-cholinergic transmitter
eNOS	epithelial nitric oxide synthase
GMP	guanosine monophosphate
GSH	glutathione
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
HO	haem oxygenase
HO^-	hydrxide ion
HO-1	haem oxygenase-1
HO-2	haem oxygenase-2
HO-3	haem oxygenase-3
HOARSI	horse owner assessed respiratory signs index
HOCl	hypochlorous acid
HRP	horseradish peroxidase
IAD	inflammatory airway disease
ICAM-1	inter-cellular adhesion molecule 1
IFN-γ	interferon-γ
IgE	immunoglobulin E
IgG	immunoglobulin G
IL-1	interleukin-1
IL-10	interleukin-10
IL-17	interleukin-17
IL-1β	interleukin-1β
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-8	interleukin-8
iNANC	inhibitory non-adrenergic, non-cholinergic system

iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
LTA_4	Lymphotoxin-α4
LTB_4	Lymphotoxin-β4
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MIP 2	cytokine MIP-2
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
MMPs	matrix metalloproteinases
MPO	myeloperoxidase
mRNA	messenger ribose nucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NADPHd	nicotinamide adenine dinucleotide phosphate diaphorase
NF-κβ	nuclear factor-κβ
nNOS	neural nitric oxide synthase
NO^-	nitroxyl anion
NO^+	nitrosonium cation
NO_2	nitrogen dioxide
NO_2*	nitronium ion
NO_2Cl	nitrous chloride
NOS-1	nitric oxide synthase-1
NOS-3	nitric oxide synthase-3
NT	nicotinamide
O_2	molecular oxygen
O_2^-	superoxide anion
$ONOO^-$	peroxonitrite anion
p	p-value
ppb	parts per billion
ppm	parts per million
PBS	phosphate buffered saline
PELF	pulmonary epithelial lining fluid
PFT	pulmonary function test
Q	perfusion
r	Pearson correlation coefficient
RAO	recurrent airway obstruction
RES	reticuloendothelial system
RNS	reactive nitrogen species
ROC	reactor operator characteristic
ROS	reactive oxygen species
RSQ	risk-screening questionnaire
SPAOPD	summer pasture-associated obstructive pulmonary disorder
SPARAO	summer pasture-associated recurrent airway obstruction
TA	tracheal aspirate
Tb	Thoroughbred
TGF-β	transforming growth factor β
Th1	T-helper type 1
Th2	T-helper type 2

TMB	3,3’,5,5’-Tetramethylbenzidine
TNCC	total nucleated cell count
TNF-α	tumour necrosis factor-α
UK	United Kingdom
USA	United States of America
V	ventilation
VCAM-1	vascular adhesion molecule 1
w	Mann Whitney statistic
Wb	Warmblood

CHAPTER 1

INTRODUCTION

1.1 Epidemiology and Aetiopathogenesis of Recurrent Airway Obstruction

1.1.1 Definition

An association between stabling and equine respiratory disease is well documented in historical texts and has likely afflicted equids for as long as they have been domesticated within confined airspaces. In 333 BC, Aristotle described a characteristic "heave" or expiratory effort associated with the condition (Smith 1919). In the last century the association between dusty or mouldy hay and occurrence of the condition was reported (Gillespie and Tyler 1969; Gerber 1973). Of the lower airway inflammatory conditions that affect horses, recurrent airway obstruction (RAO) is probably the best understood. A number of terms have been used to describe the clinical manifestations of the condition including heartache, heaves, broken wind, chronic obstructive pulmonary disease and recurrent airway obstruction (McPherson and Thomson 1983; Beech 1991). Other terms based on aetiopathogenesis have included allergic airway disease, hay sickness, chronic bronchiolitis, small airway disease and emphysema (McPherson and Thomson 1983; Beech 1991; Mair and Derksen 2000; Ainsworth and Hackett 2004). An international workshop on chronic airway disease held in Michigan in 2000 resulted in the publication of a consensus statement:

"The term 'heaves' or recurrent airway obstruction should be used for the mature horse with airway obstruction that is reversed by a change in environment or the use of bronchodilators" (Robinson 2001).

The term 'equine COPD' was derived from the human literature and used to describe the syndrome of airway obstruction, mucus accumulation and neutrophilic inflammation in the mature horse (Sasse 1971). However, human COPD is a progressive irreversible condition

generally associated with smoking and is different from ‘heaves’. The Michigan workshop concluded:

“New information in human and equine medicine has revealed major differences between human COPD and equine ‘heaves’ so that, at present, it is no longer appropriate to use the term COPD in equine medicine” (Robinson 2001).

Recurrent Airway Obstruction (RAO) is associated with housing, most commonly during the winter months. A clinically indistinguishable condition with similar clinicopathological features that occurs between spring and early autumn in horses at pasture has been termed summer pasture-associated obstructive pulmonary disease or SPAOPD (Beadle 1983). The acronym SPARAO might be considered to be more appropriate given the similarities with RAO and the absence of any clinicopathological association with human obstructive pulmonary disease. Recurrent airway obstruction and SPARAO are not mutually exclusive. Approximately 10% of horses with RAO also suffer from SPARAO and approximately 50% of horses with SPARAO demonstrate hypersensitivity to hay and straw (Mair 1996).

1.1.2 Epidemiology

Respiratory disease is the second most common cause of lost training days and retirement in racehorses after orthopaedic disease (Morris and Seeherman 1991; Rush and Mair 2004). The true prevalence of RAO is unknown; however estimates have varied from 2% to 80% depending upon inclusion criteria (McPherson *et al.* 1978; Larson and Busch 1985; Winder and von Fellenberg 1987; Bracher *et al.* 1991; Morris and Seeherman 1991). In the United Kingdom, RAO is recognised as a common cause of chronic coughing (McPherson and Thomson 1983) yet the true prevalence is unknown. From 270 horses presenting to a University Clinic in Scotland, 148 (54.8%) were diagnosed with RAO (Dixon *et al.* 1995a), however this prevalence information was derived from a population of referred horses and is unlikely to represent the true prevalence of disease. In another study of 106 cases with chronic pulmonary disease referred to a University in the South West of England, 91 were diagnosed with RAO (Mair 1987). In Switzerland, which has a climate that is broadly comparable to the United Kingdom, a prevalence of 54% was determined based upon the results of clinical examination, endoscopy, tracheal wash cytology and arterial blood gases (Bracher *et al.* 1991). Unfortunately these studies were performed prior

to publication of a consensus statement on what constituted RAO and attempts at differentiation between RAO and inflammatory airway disease (IAD) (Robinson 2001). The disease is reportedly more common in the northern hemisphere where horses are stabled through winter and fed hay cured during humid weather in the summer (Robinson *et al.* 1996). The condition is rare in the warmer, drier regions of the United States (Robinson *et al.* 1996) and has not been reported in Australia. In an abattoir survey of 166 horses in the northern United States of America (USA), 12% of horses had inflammatory lesions of the bronchi (Larson and Busch 1985).

Several studies with inconsistent inclusion criteria have reported gender or breed predilections (McPherson *et al.* 1979b; Dixon *et al.* 1995b; Seahorn *et al.* 1996; Aviza *et al.* 2001; Couetil and Ward 2003;) often with conflicting findings. Although one large retrospective study of 1,444 horses identified thoroughbreds to be at greater risk than ponies (Couetil and Ward 2003), there is no convincing evidence of an association between breed or gender and RAO. There does appear to be an association with age; most horses are 7 years of age or older at time of diagnosis (Couetil and Ward 2003; Couetil and Hinchcliff 2004; Lavoie 2007) and the mean age in one study was 12 years (Aviza *et al.* 2001).

A heritable basis for RAO has long been suspected. Only a proportion of horses kept under identical conditions will develop RAO and a high prevalence of the disease within families of horses has been reported (Gerber 1989). Marti *et al.* (1991) identified a genetic predisposition to chronic respiratory disease in populations of German Warmblood and Lippizaner horses with an increased prevalence in horses with one parent affected by the disease and a further increase in prevalence if both parents were affected. Management conditions appeared to influence development of the disease in genetically predisposed individuals demonstrating that both genotype and environment influence development of the disease. This work was supported by evidence for genetic influences on the production of allergen specific IgE thought to be via an association with genes encoding for major histocompatability complexes (MHC) (Eder *et al.* 2001).

By contrast to RAO, SPARAO most commonly occurs from late spring to early autumn in horses that are at pasture. No genetic or breed predisposition for SPARAO has been identified (Seahorn and Beadle 1993; Mair 1996) and the reported mean age of horses with the condition is 10 – 14 years (Seahorn and Beadle 1993; Rush and Mair 2004). The genetic basis of SPARAO has not been investigated.

1.1.3 Aetiology

The association between RAO and poor quality hay has been recognised for over 100 years (Cagney *et al.* 1904) and the connection between the disease and poor air quality was later established (McPherson *et al.* 1979b). Conventional equine housing systems expose horses to high levels of airborne particulates that arise from feed and bedding (Clarke 1987; Webster *et al.* 1987; Woods *et al.* 1993; Vandenput *et al.* 1997; McGorum *et al.* 1998). Stable dust may contain a myriad of agents including around 70 species of fungi and actinomycetes, numerous species of forage mites, endotoxins and inorganic components (Halls and Gudmundsson 1985; Clarke and Madelin 1987; Woods *et al.* 1993; Pirie *et al.* 2002, 2003a). The relative importance of individual agents in the pathogenesis of the disease is yet to be elucidated and has been the subject of considerable scientific investigation (Robinson 2001).

1.1.3.1 Moulds

Aspergillus fumigatus, *Faenia rectivirgula* (formerly *Micropolyspora faeni*) and *Thermoactinomyces vulgaris* are particularly abundant in poor quality hay (Woods *et al.* 1993) and have been suspected to be aetiological agents of RAO. Inhalation of *A. fumigatus* or *F. rectivirgula* has been identified to cause a neutrophilic pulmonary inflammatory response and impaired pulmonary function in horses with RAO but not control horses (McPherson *et al.* 1979a; Derksen *et al.* 1988; McGorum *et al.* 1993d). Horses with RAO have significantly higher levels of IgE to mould allergens in bronchoalveolar lavage fluid (BALF) (Halliwell *et al.* 1993; Schmallenbach *et al.* 1998; Eder *et al.* 2000) and serum (Eder *et al.* 2000) compared to control animals. Horses with RAO show a greater disease response to hay and straw exposure than to experimental mould extracts (McGorum *et al.* 1993c) suggesting that other factors present in hay/straw are also important in triggering disease. Moulds contain a number of components that may contribute to disease in humans and animals including glucans, proteases and mycotoxins (Williams 1997). In horses, hay dust suspensions with a higher concentration of β-glucan are more likely to induce airway inflammation (Pirie *et al.* 2002) and in humans, β-glucan potentiates the inflammatory response to other agents, particularly endotoxin (Williams 1997). By contrast, horses with SPARAO do not have increased circulating concentrations of mould or pollen specific IgG or IgE (Seahorn *et al.* 1997).

1.1.3.2 Endotoxin

Experimentally, endotoxin exposure induces a dose-dependent airway neutrophilia in both horses with RAO and horses with no evidence of airway disease (Pirie *et al.* 2001). In addition, airway dysfunction occurs in horses with RAO after exposure to endotoxin (Pirie *et al.* 2001). Endotoxin has the capacity to prime neutrophils and inhibit neutrophil apoptosis (Pirie *et al.* 2001). Endotoxin has also been demonstrated to have a synergistic effect with organic dust particles (particularly mould spores) in inducing neutrophilic airway inflammation (Pirie *et al.* 2003b). However, reported concentrations of endotoxin in stables in Sydney, Australia (where RAO has not been reported) were similar to those reported previously in the UK (Malikides 2004).

1.1.3.3 Mites

Forage mites have been implicated in the aetiopathogenesis of occupational asthma in humans (Cuthbert *et al.* 1979) and are present in high concentrations in the stable environment (Halls and Gudmundsson 1985; Clarke and Madelin 1987; Woods *et al.* 1993). Although their importance has been investigated, meaningful conclusions regarding their importance in the aetiopathogenesis of RAO have not been reached (Robinson 2001).

1.1.3.4 Non-specific hyperreactivity

Horses with RAO develop a generalised airway hyperreactivity (Derksen *et al.* 1985b) and are sensitive to non-specific inflammation by environmental factors such as noxious gases (ammonia, hydrogen sulphide and ammonia), inorganic dusts and cold or dry air (Lavoie 2007).

1.1.3.5 Bacterial and viral infections

Circumstantial evidence has led to the suggestion that bacterial or viral infections may predispose to the development of RAO (Gerber 1973; Lavoie 1997; Mair and Derksen 2000) however this remains to be substantiated.

1.1.4 Pathogenesis

The series of events between exposure of horses to aetiological agents and the development of clinical signs is yet to be elucidated fully; from what is currently known it would appear to be highly complex.

1.1.4.1 Inflammation

1.1.4.1.1 Hypersensitivity reactions

It is well established that IgE-mediated reactions play an important role in asthma in humans (Guo *et al.* 1994; Coyle *et al.* 1996). The finding that horses with RAO have significantly higher concentrations of IgE directed against mould allergens in bronchoalveolar lavage fluid (BALF) (Halliwell *et al.* 1993; Schmallenbach *et al.* 1998; Eder *et al.* 2000) and serum (Eder *et al.* 2000) than unaffected horses is consistent with a Type I hypersensitivity response. Alternatively, such findings could merely represent sensitisation of horses following exposure. Conversely, in one study, tracheal wash fluid from horses with SPARAO contained lower concentrations of IgE than that collected from control horses (Seahorn *et al.* 1997). Typically, clinical signs of RAO are not seen immediately following exposure of susceptible animals to allergen, suggesting an IgE mediated mast cell response (a classical Type I hypersensitivity reaction) is not central to the disease process. However, involvement of IgE and mast cells in the aetiology of RAO cannot be dismissed.

Increased concentrations of serum precipitating antibodies to *F. rectivurgula* in one study was consistent with a Type III hypersensitivity response in horses with RAO (Halliwell *et al.* 1979), however in another study increases in precipitating antibodies were not identified consistently (Lawson *et al.* 1979). Furthermore, pathological changes in RAO are not characteristic of a type III hypersensitivity response (Lawson *et al.* 1979).

1.1.4.1.2 Mast cells

The role of mast cells in the pathogenesis of RAO remains controversial. In response to allergen challenge, the concentration of histamine in pulmonary epithelial lining fluid (PELF) has been shown to increase coincident with a decrease in the number of mast cells in BALF (Derksen *et al.* 1988; McGorum *et al.* 1993c). These findings are consistent with the degranulation of mast cells in response to allergen (Derksen *et al.* 1988; McGorum *et*

al. 1993c). *In vitro*, pulmonary mast cells from horses with RAO degranulate faster in response to challenge with fungal antigens than do those from control horses, consistent with sensitisation of mast cells in horses with RAO (Hare 1999). Mast cells from both healthy and RAO-affected horses have been shown to degranulate in response to challenge with hay dust suspension *in vitro* (Dacre *et al.* 2005), however numbers of mast cells have been demonstrated to increase to a similar degree after antigen exposure in both RAO and control horses (Marlin *et al.* 2005). A recent study demonstrated that the number of chymase-positive mast cells was increased in the bronchial and bronchiolar walls of horses with RAO with a relationship being identified between numbers of chymase positive mast cells and both pulmonary fibrosis and infiltration of lymphocytes and neutrophils (van der Haegen *et al.* 2005).

1.1.4.1.3 TH1 vs TH2 lymphocyte responses

Lower airway inflammation, reversible airway obstruction and bronchial hyper-responsiveness are characteristic of both human asthma and RAO. A T helper type 2 (TH2) response is involved in the aetiopathogenesis of human asthma and it has been postulated that similar mechanisms would also be prominent in RAO (Lavoie 2007). Evidence of IgE-mediated degranulation of mast cells and the discovery that histamine levels in BALF are increased 5 hours after natural challenge (McGorum *et al.* 1993b) were consistent with TH2 response in horses with RAO. Furthermore, increased mRNA expression for interleukin-4 (IL-4) and interleukin-5 (IL-5) and decreased expression for interferon gamma (IFNγ) in BALF have been identified (Lavoie *et al.* 2001; Cordeau *et al.* 2004; Horohov *et al.* 2005) providing further evidence of a TH2 response. Interleukin-4 stimulates proliferation of the TH2 cell phenotype and induces B lymphocytes to produce IgE consistent with previous findings of increased IgE in BALF and serum following induction of disease (Eder *et al.* 2000; Halliwell *et al.* 1993; Schmallenbach *et al.* 1998). Conversely, other authors did not demonstrate characteristic TH-2 cytokine profiles in horses with RAO (Beadle *et al.* 2002; Giguere *et al.* 2002; Ainsworth *et al.* 2003; Kleiber *et al.* 2005) and failed to demonstrate a correlation between antigen-specific IgE and expression of disease (Dixon *et al.* 1996; Eder *et al.* 2000) casting doubt that the mechanism of disease is a typical TH2 response. Furthermore, IgG responses in horses with RAO identified in some studies would be more consistent with a T helper type 1 (TH1) response than a TH2 response (Halliwell *et al.* 1993; Ainsworth *et al.* 2002). The reason for discrepancies between studies is unclear; however they may include differences in challenge, differing methodologies, interaction of other non-allergic pro-inflammatory

aetiological agents or differences in time points of the disease process at which samples were collected.

1.1.4.1.4 Neutrophils

A TH-2 response and the release of increased levels of IL-5 would be expected to result in eosinophilia as occurs in human asthma; however this is an uncommon cytological finding in the airways of horses with RAO and SPARAO in which neutrophils are the predominant inflammatory cell. Blood and airway neutrophils are activated in horses with RAO (Tremblay *et al.* 1993; Marr *et al.* 1997; Pellegrini *et al.* 1998; Brazil *et al.* 2005) and accumulate in the airways of diseased animals within 6 hours of exposure to mouldy hay (Fairbairn *et al.* 1993b; Pirie *et al.* 2001; Brazil *et al.* 2005). Apoptosis of neutrophils is delayed in horses with RAO contributing to the neutrophilia in the peripheral airways (Brazil 2000; Turlej *et al.* 2001). The triggers for neutrophil migration to the airway are poorly understood, however upregulation of mRNA expression of the neutrophil chemoattractants IL-8, macrophage inflammatory protein 2 (MIP-2), IL-1β and TNF-α have been shown to be up-regulated in RAO (Franchini *et al.* 1998; Franchini *et al.* 2000; Giguere *et al.* 2002; Laan *et al.* 2005). In one study, expression of IL-8 mRNA remained increased even when horses were in remission (Giguere *et al.* 2002). Interleukin-17 (secreted by activated T-cells) promotes maturation, chemotaxis and activation of neutrophils; increased mRNA expression of this cytokine was found in horses with RAO following exposure to mouldy hay (Debrue *et al.* 2004). Recent evidence suggests an important role for airway-derived IL-8 in neutrophil chemotaxis with IL-17 not being upregulated until later in the course of disease (Ainsworth *et al.* 2006). In man some TH-2 cytokines such as IL-4 are able to modulate neutrophil kinetics hastening neutrophil maturation and delaying apoptosis (Bober *et al.* 1995; Girard *et al.* 1997).

Neutrophils produce pro-inflammatory cytokines and chemokines including TNF-α, IL-1β, IL-6, IL-8 and MIP-2 (Joubert *et al.* 2001; Giguere *et al.* 2002). Increases in mRNA expression of IL-1β, TNF-α and IL-8 are consistent with increased nuclear factor kappa beta (NF-κβ) activity in RAO (Bureau *et al.* 2000a; Bureau *et al.* 2000b; Sandersen *et al.* 2001).

Nuclear factor-κβ is activated by, and regulates expression of, a number of inflammatory cytokines (including IL-1β, TNF-α and IL-8) and is associated with resistance of inflammatory cells to apoptosis resulting in autoregulatory positive feedback loops that potentiate the inflammatory process (Barnes 1997). Nuclear factor-κβ is also activated by

endotoxin (Barnes 1997). Nuclear factor-κβ complexes identified in RAO were p65 homodimers rather than the more typical p65-p50 heterodimers; a finding that might explain the bias toward a neutrophilic rather than an eosinophilic inflammatory response (Sandersen *et al.* 2001) considering that p65 homodimers induce IL-8 production (a potent neutrophilic chemoattractant) whereas p65-p50 heterodimers induce eotaxin production (a potent eosinophilic chemoattractant). Bronchial NF-κβ activity strongly correlates to airway neutrophilia and its activity remains increased until granulocyte death (Bureau *et al.* 2000a).

Activated neutrophils may potentially contribute to the pathogenesis of RAO by a number of pathophysiological mechanisms. Neutrophils may release up to 50 histotoxins including reactive oxygen species, proteases (elastase, collagenase, metalloproteinase-9) which may contribute to tissue damage and release of pro-inflammatory mediators (LTB_4, platelet-activating factor, thromboxane A_2, LTA_4, lactoferrin, myeloperoxidase, lysozyme and nitric oxide) (Lavoie 2007). Pathologic increases in collagenolytic activity associated with increased expression and synthesis of matrix metalloproteinase-8 (MMP-8) and matrix metalloproteinase-13 (MMP-13) have been identified in horses with RAO (Koivunen *et al.* 1997a). Excessive synthesis of these enzymes may result in destruction of the extracellular matrix and basement membrane, facilitating recruitment of inflammatory cells to sites of inflammation (Riley *et al.* 1988). Gelatinolytic and elastinolytic MMPs including MMP-2 and MMP-9 are also upregulated in horses with RAO and may be involved in the pathogenesis of this disease (Koivunen *et al.* 1997b; Raulo and Maisi 1998; Raulo *et al.* 2000; Raulo *et al.* 2001; Nevalainen *et al.* 2002; Simonen-Jokinen *et al.* 2005a; Simonen-Jokinen *et al.* 2005b).

Increases in the production of neutrophil elastase 2A have also been identified in horses with RAO (Brazil 2000). Myeloperoxidase activity is increased in the BALF of horses with RAO during disease exacerbation and remission and may prove to be a marker of neutrophil activation (Art *et al.* 2006).

1.1.4.1.5 Oxidative and Nitrosative injury

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) may enter the lung by inhalation or, more commonly, are generated by endogenous enzymatic or other chemical reactions. Reactive oxygen species is a collective term for multiple free oxygen radicals such as the super oxide anion and hydroxyl radicals and derivatives of oxygen that do not contain unpaired electrons such as hydrogen peroxide, hypochlorous acid, peroxynitrite

and ozone. The products of reaction between NO and super oxide, such as peroxynitrite, NO_2Cl and NO_2* are more reactive than their precursors. Simultaneous release of the two molecules is common during inflammatory processes and enhances cytotoxicity.

Production of free radicals is an inevitable consequence of normal metabolic processes in all cells. The major function of ROS and RNS is the killing of invading micro-organisms. These molecules may be generated in granulocytes by peroxidases which catalyse the reaction of hydrogen peroxide with halides leading to the formation of hypohalides such as hypochlorous acid (Andreadis *et al.* 2003). In addition, all activated phagocytes (macrophages, monocytes, neutrophils and eosinophils) generate superoxide anions in response to activation of the membrane bound NADPH-oxidase complex by particulate matter, micro-organisms and other mediators (Henricks *et al.* 1986; Babior 2000). However, exposure of tissues to increased levels of ROS and RNS results in damage to DNA, lipids, proteins and carbohydrates, neutrophil migration and sequestration, and escalation of the inflammatory process. Considerable research has focused on the regulation of redox sensitive transcription factors during periods of oxidative stress. Nuclear factor-κβ and activation protein-1 (AP-1) are fundamental in the inflammatory response and are activated by both ROS and RNS (Barnes 1997). Furthermore, synergistic activity has been demonstrated between ROS/RNS and inflammatory mediators such as TNF-α (Bowler and Crapo 2002).

Oxidative or nitrosative stress is an imbalance between numbers and activity of oxidants and anti-oxidants. Pulmonary epithelial lining fluid and lung tissues contain enzymes and chemicals to protect against nitrosative and oxidative injury. The most important anti-oxidant enzyme systems are the copper-zinc super oxide dismutases which rapidly convert the super oxide anion to hydrogen peroxide, catalase which converts hydrogen peroxide into water and oxygen and the glutathione redox system (GSH-peroxidase and GSH-reductase) that inactivates NO, hydrogen peroxide and other hydroperoxides (Cantin *et al.* 1990; Repine *et al.* 1997; Pietarinen-Runtti *et al.* 2000). Other molecules that scavenge free radicals in the airways include vitamin E (α-tocopherol), vitamin C (ascorbic acid), uric acid, β-carotene, flavonoids, taurine, lactoferrin, albumin and bilirubin. There are marked differences between the anti-oxidant defenses in the upper and lower respiratory tract. Bronchoalveolar lavage fluid contains ceruloplasmin and transferrin and large amounts of ascorbic acid and glutathione [95% of which is in a reduced state (Cantin *et al.* 1987)] (Horvath *et al.* 2005). However, little data exists on the normal range of anti-oxidant defences in epithelial lining fluid and marked differences may occur between

normal individuals. Asthmatics have been shown to have reduced levels of ascorbic acid and tocopherol within ELF; however how this reduction relates to antioxidant defences is unknown (Bowler and Crapo 2002).

Whilst there is a huge quantity of circumstantial evidence implicating oxidative stress in the pathogenesis of inflammatory lung diseases, the hypothesis is unproven due to the absence of any potent anti-oxidants that could be used to demonstrate the importance of the oxidant/anti-oxidant balance (Horvath *et al.* 2005).

Considering the above caveat, there is considerable circumstantial evidence of the importance of ROS and RNS in the pathogenesis of pulmonary disease in man, in particular acute respiratory distress syndrome (ARDS), interstitial lung disease, cystic fibrosis, COPD and asthma (Huie and Padmaja 1993; Repine *et al.* 1997; Vallyathan and Shi 1997; Goldstein and Czapski 2000; Folkerts *et al.* 2001; Bowler and Crapo 2002). Horses with RAO have been shown to have evidence of pulmonary oxidative stress on the basis of oxidation of glutathione in the pulmonary epithelial lining fluid (PELF) (Art *et al.* 1999) and a decrease in reduced ascorbic acid in PELF and plasma (Deaton *et al.* 2004a; Deaton *et al.* 2006). However, in another study, when horses with RAO were challenged with organic dust there was no evidence of severe pulmonary oxidative stress despite ascorbic acid consumption (Deaton *et al.* 2005a).

1.1.4.1.6 Macrophages

Whilst most research has centred on the role of the acquired immune system in the pathogenesis of RAO, there is evidence of the involvement of macrophages and other antigen-presenting cells in the disease. It has been suggested that BAL macrophages from RAO-affected horses are in an increased state of activation (Tremblay *et al.* 1993) compared to control horses and exhibit an increased expression of the neutrophil chemoattractants TNF-α, IL-1β, and IL-8 (Laan *et al.* 2005; Laan *et al.* 2006).

1.1.4.1.7 Non-inflammatory cells

The importance and role of epithelial cells in the pathogenesis of RAO is poorly understood. The location of airway epithelial cells affords these cells an important role in determining responses to the environment and they are able to signal to, and receive signals from, the innate and adaptive limbs of the immune system (Ainsworth 2005). Bronchial epithelial cells of horses with heaves have been demonstrated to express

increased quantities of NF-κβ that correlate to decreased airway function and BALF neutrophilia (Bureau *et al.* 2000a; Bureau *et al.* 2000b). Bronchial epithelial cells of horses with SPARAO demonstrate increased immunoreactivity to inducible nitric oxide synthase (iNOS) (Costa *et al.* 2001) which may down-regulate the TH1 response in favour of a TH2 response.

1.1.4.2 Bronchoconstriction

The importance of bronchospam in the pathogenesis of RAO is demonstrated by the rapid improvement in clinical signs and pulmonary function following administration of bronchodilators (Murphy *et al.* 1980; Pearson and Riebold 1989; Erichsen *et al.* 1994). The precise cause of the bronchospasm is unknown and is likely multifactorial. Anticholinergic and β_2-adrenergic agonists have similar bronchodilatory effects suggesting that muscarinic receptors are important in mediating bronchospasm (Robinson 2001).

Multiple inflammatory mediators including endothelin-1, serotonin/5-HT, histamine and leukotriene D_4 (LTD_4) have the potential to induce bronchoconstriction via specific receptors (Derksen *et al.* 1985a; Klein and Deegen 1986; Benamou *et al.* 1998; Marr *et al.* 1998b; Doucet *et al.* 1990; Guthrie *et al.* 1992; Olszewski *et al.* 1997; Olszewski *et al.* 1999a; Olszewski *et al.* 1999b) however their relative importance is unknown. Histamine, 5-HT and LTD_4 increase the response of smooth muscle to acetylcholine (Ach) and histamine and 5-HT also increase the release of Ach from these nerves. Antagonism of histamine or leukotrienes fails to induce a significant clinical response suggesting that these mediators are not central to the induction of bronchoconstriction (Lavoie *et al.* 2002; Marr *et al.* 1998a; Olszewski *et al.* 1999a). Reduced inhibition of bronchoconstriction by inhibitory nonadrenergic noncholinergic nerves may also have a role (Yu *et al.* 1994).

1.1.4.3 Mucus accumulation

Mucus is a complex biofilm composed of water, electrolytes, enzymes, epithelial and inflammatory cells and mucins (high molecular weight O-linked glycoproteins) secreted by goblet cells in the surface epithelium and subepithelial glands (Robinson 2001). Mucus accumulation may be a consequence of increased secretion (Kaup *et al.* 1990), alterations

in the ciliary apparatus or increased mucus viscosity (Robinson 2001). The presence of large quantities of mucus is a consistent finding in the airways of horses with RAO (Dixon *et al.* 1995a; Robinson *et al.* 1996). Mucus produced in diseased animals is of higher viscosity and slower to clear than in control animals (Gerber *et al.* 2000) and may remain present even during periods of remission (Jefcoat *et al.* 2001). Accumulation of mucus in the lower airways is associated with airway neutrophilia and coughing (Robinson *et al.* 2003). Increased tracheal secretions are produced in horses with RAO within 5 hours of challenge (Pirie *et al.* 2001). Quantitative and qualitative changes in the carbohydrate side-chains occur in the mucins of horses with RAO (Jefcoat *et al.* 2001) which may be the result of expression of different mucin genes. One mucin in particular, eqMUC5AC appears to be upregulated in horses with RAO (Gerber *et al.* 2003b). Accumulation of mucus may result in plugging of the distal airways and ventilation-perfusion mis-matching as well as predisposing affected animals to secondary bacterial infection (Lavoie 2007).

1.1.4.4 Airway remodelling

RAO has generally been considered to be a reversible disease; however evidence exists of progressive loss of pulmonary function as the disease progresses. A relationship between pulmonary function and bronchiolar remodelling *post mortem* has been identified (Kaup *et al.* 1990). Pulmonary smooth muscle mass may increase 3-fold in horses with RAO resulting in narrowing of the peripheral airways. In human asthma, smooth muscle hypertrophy is the most important contributor to increased airway resistance and it is proposed that the process may be of similar importance in horses with RAO (Lavoie 2005). Peribronchiolar fibrosis and epithelial hyperplasia also occur in response to chronic inflammation of the airways and will contribute to a narrowing of the airways (Kaup *et al.* 1990).

1.1.5. Pathology

The results of macroscopic examination of the lungs of horses affected by RAO indicate that pathology may be variable in distribution and severity (Gerber 1973; Beech 1991). The lungs may appear normal or there may be evidence of hyperinflation and pallor (Gerber 1973; Beech 1991). With severe disease, hyperinflation may be evident as emphysema, however this finding must be interpreted with caution as it may be identified

in older horses in the absence of respiratory disease (Thurlbeck and Lowell 1964). An increased quantity of exudate is commonly present within the airways.

Histological abnormalities of RAO are most notable within the distal airways. Mucus, neutrophils and other cellular debris are present within the airways often coalescing to form pools of exudate and mucin plugs (Thurlbeck and Lowell 1964; Kaup *et al.* 1990). The distal airways show evidence of smooth muscle hypertrophy, lymphocytic and plasmacytic infiltration, epithelial metaplasia, desquamation, goblet cell hyperplasia/metaplasia and fibrosis (Thurlbeck and Lowell 1964; Winder and von Fellenberg 1987; Kaup *et al.* 1990). Eosinophilia is not a consistent finding in contrast to the findings of examination of the distal airways from patients with asthma (Barnes 1987). Bronchiectasis may result in separation of the cartilaginous plates of the dilated bronchi, disruption of chondrocyte arrangement and a reduction in the number of elastic fibres in the lamina propria (Lavoie *et al.* 2004). It has been reported that fibrosis and emphysema are not prominent findings in horses with SPARAO (Costa *et al.* 2000b), however this is possibly due to the smaller case numbers that have been examined.

1.2 Epidemiology and Aetiopathogenesis of Inflammatory Airway Disease

1.2.1 Definition

The term “inflammatory airway disease” (IAD) was proposed in 2000 to distinguish a less severe form of respiratory tract inflammation from recurrent airway obstruction (Robinson 2001). In 2002, a workshop entitled “Inflammatory Airway Disease: defining the syndrome” was held to clarify what was known of the clinical presentation, epidemiology, diagnosis, functional consequences and pathology of the syndrome (Robinson 2003). Further research into the syndrome followed and in 2007 the American College of Veterinary Internal Medicine published a consensus statement in order to further clarify the distinctions between IAD and RAO (Couëtil *et al.* 2007). The authors of the consensus statement proposed the following minimum criteria to define the IAD phenotype in horses of any age:

- Poor performance, exercise intolerance, or coughing, with or without excess tracheal mucus.

- Nonseptic inflammation detected by cytologic examination of bronchoalveolar lavage fluid (BALF) or pulmonary dysfunction based on evidence of lower airway obstruction, airway hyper-responsiveness, or impaired blood gas exchange at rest or during exercise.

Further, the authors proposed the following exclusion criteria:

- Evidence of systemic signs of infection (fever, haematologic abnormalities compatible with infection).
- Increased respiratory effort at rest (i.e., heaves) (Couëtil *et al.* 2007)

The authors of the consensus statement acknowledged that tracheal inflammation and infection are prevalent in horses, especially young racehorses (Christley *et al.* 2001; Wood *et al.* 2005a) and that the narrow inclusion criteria proposed in the statement excluded these cases. The link between such cases, seen commonly in the United Kingdom, and IAD as defined above remains to be established. This and other controversies, most notably differing methods of diagnosis, definitions of cytological findings and the validity of TA findings have prevented the above recommendations from achieving universal acceptance.

1.2.2 Epidemiology

The prevalence and incidence of IAD is likely to vary between differing populations of horses and comparison between studies is difficult due to differing inclusion criteria. Different sub-categories of IAD with different aetiologies likely exist and their prevalence is likely to depend upon the equine population under investigation (Christley and Rush 2007). Prevalence of IAD in young racehorses is high and studies using mucus scores and/or airway neutrophilia in Thoroughbreds and Standardbreds have resulted in a prevalence of 11-50% (Burrell 1985; MacNamara *et al.* 1990; Sweeney *et al.* 1992a; Chapman *et al.* 2000). In older horses, prevalence is less well studied, however it is reported that prevalence may be up to 70% in horses maintained permanently indoors (Robinson 2003). Incidence of IAD is also high especially in horses entering training. In one study of Thoroughbred racehorses, 41% of horses entering a training yard developed respiratory disease within 2 weeks (Malikides 2004). In the UK, incidence of IAD was reported to be 8.9 cases/100 horses/month (Wood *et al.* 2005b). Among young racehorses the risk of IAD decreases with age (Chapman *et al.* 2000; Christley *et al.* 2001; Wood *et*

al. 2005b) which may indicate tolerance to the change of environment or the development of immunity to micro-organisms.

1.2.3 Aetiology

A variety of infectious and/or non-infectious agents may be involved in the aetiology of IAD and various interactions maybe required for development of disease. It is likely that the relative importance of aetiological agents is population dependent and determined by feeding, housing, preventive medicine practices, differences in distribution of infectious agents and genetic influences (Couëtil *et al.* 2007). The introduction of horses to a stable environment has been identified as a risk factor for the development of IAD (Tremblay *et al.* 1993; Holcombe *et al.* 2001) however, little is known of the relative importance of different respirable allergens and irritants. Airborne particulates may be ten times more concentrated in a stable environment, compared to outdoor air and 30-40% of the particulate matter may be sufficiently small to reach the pulmonary alveoli (Christley and Rush 2007). Stable management practices such as cleaning and feeding and horse movement may further increase the concentration of airborne particulates within the breathing zone (Christley and Rush 2007). Coughing, a sign of respiratory inflammation, persists for longer in a high dust environment (Burrell *et al.* 1996). The presence of increased numbers of pulmonary mast cells and eosinophils in BALF from horses with IAD has been proposed to indicate the involvement of aeroallergens in development of the disease (Hare and Viel 1998; Hoffman *et al.* 1998), however specific immunological mechanisms have yet to be elucidated. Endotoxin has been studied as a major pro-inflammatory component of particulate matter. Inhaled endotoxin will induce airway inflammation in horses formerly free of respiratory disease (Pirie *et al.* 2001) and the concentration of endotoxin in the breathing zone is associated with the development of IAD in young racehorses (Malikides 2004). Other inhalants such as sulphur dioxide, nitrogen dioxide, ozone, carbon monoxide, ammonia, hydrogen sulphide, methane, glucan, ultrafine organic and inorganic dust particles, micro-organisms and mite debris are all present within the stable environment and may contribute to airway inflammation (Christley and Rush 2007).

Transportation is also associated with lower respiratory tract inflammation (Raidal *et al.* 1995; Moore *et al.* 1996). Concentrations of aeroallergens can be as high in transport vehicles as they are in the stable environment. Head elevation during transit results in increased penetration of inhalants into the lower respiratory tract; as a result of reduced mucociliary clearance (Racklyeft and Love 1990). Transport stress may also result in

immunosuppression and reduction in pulmonary defence mechanisms (Raidal *et al.* 1995; Raidal *et al.* 1997).

High intensity exercise is associated with respiratory tract inflammation (McCarthy *et al.* 1991; Moore *et al.* 1996; Christley *et al.* 2001). The reason for this is unknown, however exercise results in increased inhalation of particulate matter, increased exposure of the distal airways to unconditioned air and exercise-induced pulmonary haemorrhage, all of which may contribute to development of inflammation of the lower airways (Christley and Rush 2007). Exercise is also reported to adversely affect immune function by compromising pulmonary macrophage and peripheral lymphocyte function and increasing serum cortisol and pulmonary oxidative stress (Raidal *et al.* 2000; Raidal *et al.* 2001).

The role of infectious agents in the aetiopathogenesis of IAD is unclear. Whilst there appears to be a clear association between presence of bacteria in the trachea and inflammation it remains to be clarified whether they are causal or merely represent secondary opportunistic colonisation (Viel 1997; Christley *et al.* 2001). In several studies performed in the United Kingdom and Australia, tracheal inflammatory changes were found to be positively correlated with the number of bacterial colony forming units (CFU) in tracheal aspirates (Burrell 1985; Burrell *et al.* 1996; Chapman *et al.* 2000; Christley *et al.* 2001; Wood *et al.* 2005a, 2005b). In longitudinal studies performed in the UK, 80% of horses with IAD had in excess of 10^5 CFU per millilitre of tracheal aspirate fluid and tracheal washes failed to yield bacterial growth in only 4% of horses with IAD (Wood *et al.* 2005a). The bacterial species most frequently isolated from tracheal aspirate samples obtained from horses with IAD are *Streptococcus zooepidemicus, Streptococcus pneumoniae*, *Pasteurella* spp., *Mycoplasma felis* and *Mycoplasma equirhinis* with mixed infections being common (Burrell 1985; Burrell *et al.* 1996; Chapman *et al.* 2000; Christley *et al.* 2001; Wood *et al.* 2005a, 2005b). Similarly, in a large study performed in Australia, Thoroughbred racehorses which were coughing were more likely to have intracellular bacteria identified in tracheal aspirate samples (Christley *et al.* 2001). Conversely, bacterial involvement in horses with IAD in North America is reported less frequently (Robinson *et al.* 2006; Bedenice *et al.* 2008). The reason(s) for the geographical differences are not clear; however influences of study design, horse population differences and environmental differences may be involved.

The role of viruses in the aetiopathogenesis of IAD is unclear also. While antibodies to equine herpes viruses and equine influenza virus have been detected in horses with chronic pulmonary inflammation, Burrell *et al.* (1996) and Christley *et al.* (2001) did not find an

association between IAD and viral seroconversion. However, viruses may inhibit respiratory defence mechanisms and exacerbate airway hyper-responsiveness and hence may be a component of multi-factorial disease (Christley and Rush 2007). In a population of Standardbred racehorses, oral administration of interferon-α, an immune stimulant with anti-viral activity, was associated with a reduction in lower respiratory tract inflammation in racehorses with IAD (Rush 1997).

1.2.4 Pathogenesis

Given the lack of clear understanding of the potential aetiologies if IAD it is inevitable that the pathogenesis is similarly unclear. Couetil *et al.* (2007) defined IAD as a disease that "is characterised by nonseptic inflammation detected by cytologic examination of BALF or pulmonary dysfunction based on evidence of lower airway obstruction, airway hyper-responsiveness, or impaired blood gas exchange at rest or during exercise". It is likely that there are multiple innate and adaptive mechanisms important to the pathogenesis of IAD. Although the presence of inflammation detected by cytologic examination of tracheal aspirates is considered by some authors to be insufficient to diagnose IAD (Derksen *et al.* 1989; Holcombe *et al.* 2006; Robinson *et al.* 2006), increased total nucleated cell counts (TNCC) with increased proportions of neutrophils, eosinophils and mast cells are commonly observed in IAD (Burrell 1985; Christley *et al.* 2001; Wood *et al.* 2005b). Inflammatory airway disease also results in an increased TNCC in BALF with mild neutrophilia, lymphocytosis and monocytosis (Fogarty and Buckley 1991; Rush Moore *et al.* 1995; Couetil *et al.* 2001). Increases in mast cell and eosinophil counts are also detected in some horses with IAD (Hare *et al.* 1994; Hare and Viel 1998; Hoffman 1999). The presence of increased numbers of eosinophils and mast cells may suggest a type I hypersensitivity response. In one study, horses with IAD had increased numbers of CD8+ lymphocytes in their airway secretions whereas RAO horses had increased numbers of CD4+ lymphocytes (Moore *et al.* 1995). It has also been suggested that IAD is neither an allergic disorder nor an early stage of RAO (Moore *et al.* 1995). The immunological basis to IAD is yet to be elucidated.

Horses with IAD do not exhibit signs of respiratory dysfunction at rest, however they may exhibit abnormal responses to pulmonary function testing and reduced exercise tolerance (Couëtil *et al.* 2007). Accumulation of mucoid secretions may cause ventilation perfusion mis-match and impaired gaseous exchange (Couetil and Denicola 1999; Sanchez *et al.* 2005) and result in reduced athletic performance (MacNamara *et al.* 1990; Holcombe *et al.*

2006). The presence of bronchoconstriction and airway hyper-responsiveness are also likely to play a role in the pathogenesis of the disease as is the case with RAO; although the changes are less severe in IAD (Couëtil *et al.* 2007).

1.2.5 Pathology

The pathology of IAD has not been studied specifically. The pathological changes are assumed to be similar, but less severe, than in RAO with peribronchial inflammation, accumulation of mucoid secretions with plugging of distal airways and bronchoconstriction (Christley and Rush 2007).

1.3 Haem Oxygenase Expression in the Respiratory Tract

1.3.1 The Isoforms of Haem Oxygenase

In 1968, Tenhunen and coworkers determined that the enzyme haem oxygenase (HO) was responsible for the degradation of haem to equimolar concentrations of iron, biliverdin and carbon monoxide by cleavage of the α-meso carbon bridge of haem (Tenhunen *et al.* 1968). The presence of NADPH and molecular O_2 were determined to be required for this reaction (Tenhunen *et al.* 1968, 1969). Two further isoforms of HO that are products of different genes have since been discovered. The second form of HO (HO-2) is constitutively expressed in most tissues; highest concentrations are found in the brain, testis and liver (Maines *et al.* 1986). The third isoform (HO-3) was discovered recently and has been identified in the brain, kidney, liver, heart, testis and spleen (McCoubrey *et al.* 1997). The activity of HO-3 is poorly characterised but it is reported to have poor haem degrading capacity (McCoubrey *et al.* 1997). All 3 isoforms of HO are located intracellularly; the enzymes are anchored to the endoplasmic reticulum with extensions into the cytoplasm.

Haem oxygenase-1 is found at highest concentrations in the spleen and liver where it is important for the breakdown of haem released by erythrocyte destruction (Abraham *et al.* 2006). Haem oxygenase-1 is also known as heat shock protein 32 (Maines 1997). The heat

shock proteins are a group of highly conserved proteins that have been identified in virtually every prokaryotic and eukaryotic cell and are produced in response to heat stress or other events that are noxious to the cell (Wheeler and Wong 2007). Haem oxygenase is also known as the enzyme responsible for one of the best known colorimetric reactions in nature; namely the degradation of haem (purple) to biliverdin (green) and finally bilirubin (yellow) following sub-cutaneous haemorrhage or "bruising" (Otterbein and Choi 2000).

1.3.2 Regulation of HO-1 Expression

Haem oxygenase-2 is only expressed in response to a limited number of agents; opiates and adrenal glucocorticoids are the most important (Li and David-Clark 2000; Liu *et al.* 2000). In contrast, HO-1 is expressed in low concentrations by most cells under physiological conditions. Haem oxygenase-1 is highly active even at low substrate concentrations and activity increases dramatically in response to increased substrate concentration. To the author's knowledge Haem oxygenase-3 gene expression has not been characterised.

Haem oxygenase-1 gene expression is induced via a diverse range of signalling pathways (Maines 1997) and notably it is induced strongly by agents and conditions associated with oxidative stress. In addition, a number of important redox-independent pathways are also involved in up-regulating HO-1 gene expression. These include the mitogen-activated protein kinases (Elbirt *et al.* 1998; Oguro *et al.* 1998), protein kinase A (Immenschuh *et al.* 1998), cGMP-dependent protein kinase G (Polte *et al.* 2000) and protein phosphatases (Immenschuh and Ramadori 2000). The following agents have been identified as inducers of HO-1 expression:

Table 1.3.2 Inducers of HO-1 expression and references in the literature. Modified from Wagener et al. (2003)

HO-1 Inducer	***References***
Angiogenesis	*(Deramaudt et al. 1998; Nishie et al. 1999)*
Cytokines	*(Abraham et al. 1988; Cantoni et al. 1991)*
Endotoxin	*(Camhi et al. 1995; Otterbein et al. 1995; Carraway et al. 1998)*
Growth Factors	*(Durante et al. 1999)*
Heat Shock	*(Shibahara et al. 1987; Stuhlmeier 2000)*
Heavy Metals	*(Eyssen-Hernandez et al. 1996; Elbirt et al. 1998)*
Haem	*(Tenhunen et al. 1968, 1969; Bakken et al. 1972)*
Hyperoxia	*(Choi et al. 1995; Lee et al. 1997; Dennery et al. 1998; Taylor et al. 1998)*
Hypoxia	*(Carraway et al. 2000; Motterlini et al. 2000; Panchenko et al. 2000)*
H_2O_2	*(Keyse and Tyrrell 1987, 1989; Lautier et al. 1992)*
Nitric Oxide	*(Datta and Lianos 1999; Doi et al. 1999; Hara et al. 1999; Polte et al. 2000; Willis 1995; Wang et al. 2003)*
Phorbol Esters	*(Abraham et al. 1988; Kurata and Nakajima 1990; Alam and Den 1992)*
Prostaglandins	*(Koizumi et al. 1992; Rossi and Santoro 1995)*
Shear Stress	*(Wagner et al. 1997)*
Sodium Arsenite	*(Sardana et al. 1981; Elbirt et al. 1998)*
GSH depletion	*(Ewing and Maines 1993; Oguro et al. 1996)*
Ultraviolet A	*(Keyse and Tyrrell 1987; Ossola and Tomaro 1998)*

Haem oxygenase-1 is the major stress protein induced by hydrogen peroxide, ultraviolet radiation and sodium arsenite (Keyse and Tyrrell 1989). In addition, any agents that directly or indirectly generate ROS or alter the redox equilibrium will induce HO-1 expression. HO-1 is therefore a general marker of oxidative stress (Applegate *et al.* 1991).

Ultraviolet radiation stimulates chromophores to produce ROS (Tyrrell 1996) and the induction of HO-1 mRNA and protein has been demonstrated following cellular exposure to photosensitisers (Basu-Modak and Tyrrell 1993; Gomer *et al.* 1991; Ryter and Tyrrell 1998). Substances such as sodium arsenite and heavy metals that complex with intracellular reduced glutathione result in increased HO-1 expression (Ryter *et al.* 2002).

Nitric oxide reacts with thiols to form *S*-nitrosothiols and with O_2^- to form peroxynitrite (section 1.4). A number of studies have demonstrated that exogenous NO and NO donors activate HO-1 (Marquis and Demple 1998; Bouton and Demple 2000; Chen and Maines 2000) suggesting that there are complex interactions between different systems of anti-oxidant defence.

Inflammation, infection, sepsis and endotoxaemia all result in ROS production and in these conditions HO-1 induction may be protective via the biological roles of its products (section 1.3.4) (Rizzardini *et al.* 1993; Rizzardini *et al.* 1994). *In vitro,* the pro-inflammatory cytokines IL-1, IL-6 and TNF-α induce HO-1 expression (Rizzardini *et al.* 1993; Terry *et al.* 1999). In addition, growth factors (including transforming growth factor-β and platelet derived growth factor) induce HO-1 expression in association with the generation of ROS (Kutty *et al.* 1994; Durante *et al.* 1999).

In addition to cytokine and growth factor influences on HO-1 expression, hypoxia alters the iron and intracellular redox equilibrium activating HO-1 gene expression (Motterlini *et al.* 2000). Conversely, hyperoxia is associated with increased production of mitochondrial ROS and oxidative lung injury (Freeman and Crapo 1981).

The p38 Mitogen Activated Protein Kinase Pathway (MAPK), extracellular regulated kinases (ERK) pathway and tyrosine kinase pathways have all been variously implicated as signal transduction pathways important in the induction of HO-1 (Alam *et al.* 2000; Chen and Maines 2000; Elbirt *et al.* 1998); however the precise importance of different transduction pathways remains to be elucidated fully (Ryter *et al.* 2002).

HO-1 gene induction is inhibited by the reduction of oxidative stress; scavengers of ROS act as negative regulators (Lautier *et al.* 1992). Other biologically active molecules that inhibit HO-1 include angiotensin II (Ishikawa *et al.* 1997), interferon –γ (Takahashi *et al.* 1999), prostaglandin E_2 (Tetsuka *et al.* 1995), transforming growth factor-β (Pellacani *et al.* 1998) and IL-10 (Immenschuh *et al.* 1999).

1.3.3 Expression of HO-1 in the Lower Respiratory Tract

In the face of acute inflammation, HO activity initially decreases with the highest levels of expression being recorded as inflammation is resolving (Willis *et al.* 1996). Willis *et al.* (1996) speculated that during tissue inflammation there may be sequential induction of

enzymes with pro-inflammatory effects, for example cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), followed by increased expression of enzymes with anti-inflammatory effects, including HO-1, which are responsible for the resolution of inflammatory lesions and aimed at preventing a chronic inflammatory response.

In human airways the distribution of HO-1 has been investigated using immunostaining and western blot techniques. In one study, HO-1 was widely distributed in the airway submucosa, particularly the airway epithelium and submucosal macrophages (CD68+) as determined by double immunostaining (Brunetti *et al.* 2006). There was marked staining in alveolar macrophages from both normal and asthmatic humans and there was no statistical difference between the 2 groups (Brunetti *et al.* 2006). In a further study of normal patients and smokers, immunostaining was observed in the alveolar spaces whereas there was virtually no staining in the bronchial epithelium and vessels (Maestrelli *et al.* 2001). In that study, the majority of HO-1 positive cells were macrophages. Smokers showed an overall increase in the numbers of alveolar macrophages and an increase in the number of HO-1 positive macrophages (Maestrelli *et al.* 2001). Further evidence for macrophages being the predominant source of HO-1 was provided in a mouse model of asthma with immunofluorescence occurring mostly in the cytoplasm and particularly in the perinuclear region (Kitada *et al.* 2001). In another study, no HO-1 reactivity was detected in healthy lung tissue from mice (Minamino *et al.* 2001). In a model of inflammation in the pleural cavity, western blot analysis revealed an increase in HO-I protein in macrophages but not in neutrophils (Willis *et al.* 1996). Haem oxygenase-1 expression has been demonstrated in alveolar macrophages, neutrophils and type II epithelial cells from normal equine lungs (Wyse *et al.* 2006). Wyse *et al.* (2006) found detectable activity in both the cranioventral and caudodorsal lung lobes; with activity being comparable between the 2 regions and the level of activity being intermediate between that reported for healthy rats (Carraway *et al.* 1998) and mice (Minamino *et al.* 2001).

In one study there was no difference in HO-1 immunostaining between normal humans and patients with asthma and there was also no significant change following treatment with inhaled corticosteroids (Brunetti *et al.* 2006). A glucocorticoid response element is present in the HO-1 promoter, and gene expression of HO-1 is inhibited by corticosteroids *in vitro* (Cantoni *et al.* 1991; Lavrovsky *et al.* 1996). The reduction in iNOS expression known to result from corticosteroid therapy would be expected to result in reduced expression of HO-1 (Saleh *et al.* 1998).

1.3.4 Physiological and Cytoprotective Roles of HO-1 and its Products

The strong adaptive response of HO-1 and diverse array of stress stimuli have been interpreted to suggest roles for HO-1 in addition to haem degradation (Willis *et al.* 1996). Haem oxygenase-1 is highly conserved between species suggesting a vital role in cellular homeostasis (Lee *et al.* 1996). Haem oxygenase enzymes perform a vital physiological role in the turnover of haemoglobin-haem during the breakdown of senescent erythrocytes in reticuloendothelial cells and in the turnover of intracellular haemoproteins and cytochromes, thereby ensuring redistribution of haem and iron to the tissues (Maines 1988; Poss and Tonegawa 1997).

A cytoprotective function for HO-1 in the face of oxidative stress was first identified by (Keyse and Tyrrell 1987, 1989) with an increasing body of supporting evidence having been accumulated subsequently in various models. Haem oxygenase-1 induction by haemoglobin infusion was demonstrated to be protective in a rat model of lipopolysaccharide induced inflammatory lung injury (Otterbein *et al.* 1995). The protective role of HO-1 has also been demonstrated in multiple experiments utilising homozygous HO-1. Knockout mice showed increased mortality in the face of pulmonary ischaemia-reperfusion (Fujita *et al.* 2001), increased vascular hyperplasia in the face of arterial injury (Duckers *et al.* 2001) and increased myocardial infarction in the face of hypoxia (Yet *et al.* 1999). Chemical induction of HO-1 has been shown to be protective against hypertension and inflammation in the face of induced hypoxia in rats and mice (Christou *et al.* 2000; Minamino *et al.* 2001). Haem oxygenase-1 was protective against lung apoptosis and inflammation during hyperoxia in rats (Otterbein *et al.* 1999a).

The importance of HO-1 to human health was demonstrated when the first known human case of HO-1 deficiency was diagnosed in a 6-year old boy. The boy died having suffered growth retardation, anaemia, leukocytosis, thrombocytopaenia, coagulopathy, increased blood concentrations of haptoglobin, ferritin, haem and lipid and a decreased serum bilirubin concentration (Kawashima *et al.* 2002; Yachie *et al.* 1999). At necropsy, findings were similar to those in HO -1 knockout mice; amyloid deposits, foamy macrophages, fatty streaks and fibrous plaques were identified (Wagener *et al.* 2001).

In vitro studies in which HO-1 was produced in excess of physiological requirements provided evidence of conferred protection against haem and haemoglobin-mediated toxicity (Abraham *et al.* 2006), resistance to glutamate toxicity (Bureau *et al.* 2000b), resistance to hyperoxia (Lee *et al.* 1996; Suttner *et al.* 1999), inhibition of TNF-α induced apoptosis (Petrache *et al.* 2000) and protection against ultraviolet radiation (Vile *et al.* 1994).

The mechanisms by which HO-1 exerts its cytoprotective effects are incompletely understood. It has been suggested that a number of mechanisms may be important and differential mechanisms may exist according to the inciting injury (Zampetaki *et al.* 2003). A possible candidate for the regulation of many of these effects is NF-κβ (Zampetaki *et al.* 2003). The products of HO activity; iron, ferritin, biliverdin, bilirubin and CO have specific roles in cellular defences. By removing haem and synthesising CO and bilirubin, HO is removing a pro-oxidant compound and synthesising anti-oxidant molecules. As well as providing protection at cell level, the shift in the oxidant-antioxidant balance provides protection by reducing the expression of adhesion molecules, especially ICAM-1, and thereby reducing trafficking of activated leukocytes (Hayashi *et al.* 1999; Vachharajani *et al.* 2000).

1.3.4.1 Haem

Haem (or ferroprotoporphyrin IX) is an essential component of numerous functional proteins such as haemoglobin, myoglobin, nitric oxide synthase (NOS), cytochromes, cyclooxygenase, catalases and peroxidises (Abraham *et al.* 1988; Wagener *et al.* 2003). These proteins are fundamental in processes such as energy production, gene induction, transcription and translation, cell differentiation and proliferation and protein synthesis, splicing and modification (Wagener *et al.* 2003). The requirement for haem varies between cells and tissues but it is probable that all cells have a free haem pool responsible for regulatory functions in addition to acting as a precursor for other molecules (Wagener *et al.* 2003). The highest rates of haem synthesis occur in haemopoietic tissues such as the spleen, liver and bone marrow (Abraham *et al.* 2006). Haem turnover is high in these tissues where it is continually released from senescent erythrocytes. Haem derived from proteins other than haemoglobin is thought to be degraded in the tissues where it is produced but the majority of haemoglobin denaturation takes place within the organs of the reticuloendothelial system (RES) (Wagener *et al.* 2003).

Haem has important effects on gene expression and protein synthesis and also regulates differentiation and proliferation of various cell types (Wagener *et al.* 2001). In addition to these positive physiological effects, haem catalyses the formation of ROS thereby inducing oxidative stress and cell damage (Jeney *et al.* 2002). Haem is highly lipophilic and intercalates readily with the lipid membranes of cells and intracellular organelles, thereby rendering them unstable (Balla *et al.* 1991). Exposure to haem *in vitro* has been observed to stimulate the expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin on endothelial cells *in vitro* probably via the formation of ROS (Wagner *et al.* 1997). Activation of these adhesion molecules results in the recruitment of leukocytes to the surrounding tissues and upregulation of the inflammatory response. During *in vivo* studies, the administration of haem resulted in increased vascular permeability, adhesion molecule expression and migration of leukocytes from the vascular compartment (Wagener *et al.* 2001).

There are a number of endogenous mechanisms to reduce free haem, suggesting it has an important role in the inflammatory process (Wagener *et al.* 2003). Intracellular proteins such as haem binding protein 23, HO-2 and HO-3 and extracellular proteins such as albumin, haemopexin and HO-1 scavenge free haem (Muller-Eberhard and Fraig 1993; Maines 1997). Haptoglobin transports free vascular haemoglobin to the RES (Muller-Eberhard and Fraig 1993). Free vascular haem is transported to the liver bound to albumin or haemopexin (Muller-Eberhard and Fraig 1993).

1.3.4.2 Iron

Free iron has two free electrons and is therefore a powerful pro-oxidant capable of reacting with organic molecules to generate ROS via the Fenton reaction. To protect against these effects, iron is sequestered into the iron storage protein ferritin. The sequestration of iron as ferritin has been demonstrated to lower the pro-oxidant state of cells and the induction of ferritin was cytoprotective in a model of oxidative stress (Balla *et al.* 1992). Ferritin concentrations increase in the presence of oxidative stress (Vile and Tyrrell 1993) and furthermore increase and decrease in response to HO-1 induction and inhibition, respectively (Eisenstein *et al.* 1991). In addition, HO-1 upregulates an iron ATPase in the endoplasmic reticulum to limit levels of intracellular free iron (Baranano *et al.* 2000).

Reduction of intracellular free iron has been suggested as an explanation for the anti-apoptotic effects of HO-1 (Chen *et al.* 2000; Petrache *et al.* 2000). Over-expression of HO-1 in endothelial cells (Soares *et al.* 1998) and fibroblasts (Petrache *et al.* 2000) protects

them from TNF-α mediated apoptosis. Proposed mechanisms for this protective effect are induction of ferritin synthesis and upregulation of iron ATPase activity in the membrane of the endoplasmic reticulum, limiting cytoplasmic iron concentration (Ferris *et al.* 1999; Chen *et al.* 2000; Petrache *et al.* 2000).

1.3.4.3 Bilirubin

Biliverdin generated by HO is rapidly reduced to bilirubin by the enzyme biliverdin reductase to facilitate its excretion within bile. The importance of bilirubin as an antioxidant was recognised following the observation that bilirubin and biliverdin were highly efficient at scavenging peroxyl radicals, thereby inhibiting lipid peroxidation *in vitro*, even at micromolar concentrations (Stocker *et al.* 1987). In liposomes, bilirubin suppressed oxidation more effectively than either α-tocopherol or vitamin E.

Bilirubin is now recognised as the most abundant endogenous antioxidant in mammalian tissues and the dominant antioxidant in human serum (Gopinathan *et al.* 1994). In studies utilising models of ischaemic heart injury and oxidative damage, the administration of bilirubin was shown to be cytoprotective (Ishikawa *et al.* 1997; Dore *et al.* 1999; Clark *et al.* 2000; Vachharajani *et al.* 2000). These effects have been shown to be independent of HO-1, carbon monoxide (CO) and ferritin (Vachharajani *et al.* 2000). In guinea pig tracheal muscle, bilirubin decreases smooth muscle contractility via a reduction in intracellular ROS (Samb *et al.* 2001). Oxidative stress and airway inflammation are reduced when HO-1 expression and subsequent bilirubin production are induced by haem (Almolki *et al.* 2004).

Haem oxygenase-1 activity decreases inflammatory cell rolling, adhesion and migration from the vascular compartment (Wagener *et al.* 2001). *In vivo* evidence for the role of bilirubin in the inhibition of leukocyte rolling following oxidative stress indicated an anti-inflammatory role for bilirubin in addition to its anti-oxidant activity (Hayashi *et al.* 1999). Superfusion of bilirubin decreased leukocyte recruitment when HO-1 was inhibited with zinc protoporphyrin-IX, suggesting a role for bilirubin in leukocyte trafficking independent of HO-1. Other anti-inflammatory functions of bilirubin include increased stability of mast cell membranes resulting in reduced degranulation (Hayashi *et al.* 1999) and decreased TGF-β expression (Wang *et al.* 2002). Bilirubin also has inhibitory effects on contractility (Samb *et al.* 2001) and proliferation (Taille *et al.* 2001) of smooth muscle.

1.3.4.4 Carbon Monoxide

Carbon Monoxide (CO) is a ubiquitous atmospheric pollutant with a strong affinity for haemoglobin, forming carboxyhaemoglobin, making it a lethal agent at high concentrations due to its disruption of oxygen transport. A considerable body of evidence now indicates that at lower concentrations CO exerts important physiological functions. In man, HO generates in excess of 86% of the body's CO making it the primary source of endogenous CO (Ryter *et al.* 2002). CO exerts its physiological effects by binding to the haem moiety of guanylyl cyclase resulting in cGMP generation (Cardell *et al.* 1998a). By activating this messenger system, CO has the potential to mediate numerous physiological processes. Guanylyl cyclase is a common mediator to both CO and NO in modulating smooth muscle relaxation and vasodilation. However, CO is 30 -100 times less potent than NO (Furchgott and Jothianandan 1991). Physiological roles for CO include neurotransmission, vasodilation, bronchodilation, inhibition of platelet aggregation and anti-proliferative effects on smooth muscle (Furchgott and Jothianandan 1991; Morita *et al.* 1995; Maines 1997). Carbon monoxide has functional as well as structural similarities with NO therefore. However, unlike NO, CO is not a radical, is chemically very stable and reacts exclusively with haem. It may therefore reach higher intracellular concentrations than NO, particularly in the face oxidative stress and take over the role of NO signalling under oxidative conditions when HO-1 is induced and the bioavailability of NO is decreased (Ryter *et al.* 2002; Wagener *et al.* 2003).

1.3.4.4.1 Vascular Effects

Modulation of cGMP levels affects vascular function by influencing vasomotor tone, platelet aggregation, coagulation and smooth muscle proliferation (Morita *et al.* 1995; Motterlini *et al.* 1998; Sammut *et al.* 1998). The vasodilatory effect of CO may be important for maintaining tissue perfusion in the lungs during normal and hypoxic states (Morita *et al.* 1995). Haem oxygenase-1 knockout mice exhibited a maladaptive response to chronic hypoxia and developed thrombi and hypertension (Yet *et al.* 1999). Carbon monoxide may also dilate blood vessels by direct activation of calcium-dependent potassium channels (Koehler and Traystman 2002).

1.3.4.4.2 Anti-inflammatory effects

Carbon monoxide acts as an anti-inflammatory mediator by selectively activating several p38 mitogen-activated protein kinase (MAPK) signalling pathways independent of guanylyl cyclase (Otterbein *et al.* 2000). It is improbable that CO directly activates any one of the p38 family members and the precise biochemical mechanisms by which CO modulates the MAPKs are not clear. One hypothesis is that a haem-containing protein acts

as a proximal effector and initiates the signal upon binding to CO (Ryter and Otterbein 2004). Both *in vivo* and *in vitro*, low concentrations of CO differentially and selectively inhibited expression of the LPS-induced pro-inflammatory cytokines tumour necrosis factor-α, interleukin-1β and macrophage inflammatory protein-1β (Otterbein *et al.* 2000). Expression of the anti-inflammatory cytokine interleukin-10 (IL-10) was also increased (Otterbein *et al.* 2000). In addition, it has been suggested that IL-10 mediates many of its anti-inflammatory effects via induction of HO-1 (Giguere *et al.* 2002). Inhibition of platelet aggregation and activation through the activation of guanylyl cyclase and subsequent cGMP generation may also contribute to the anti-inflammatory effects of CO (Otterbein *et al.* 2000).

1.3.4.4.3 Effects in Oxidative Lung Injury

Carbon monoxide protects against the lethal and inflammatory hyperoxic lung injury by down-regulation of the expression of pro-inflammatory cytokines. In a model of hyperoxia-induced lung injury the presence of CO prolonged survival, likely as a result of its anti-inflammatory actions (Ryter *et al.* 2002). Carbon monoxide inhibited the appearance of markers of lung injury (haemorrhage, fibrin deposition, oedema, protein accumulation in the airway) and oxidative damage (lipid peroxidation) (Ryter *et al.* 2002). Carbon monoxide also inhibited the influx of neutrophils in BALF (Ryter *et al.* 2002). Carbon monoxide production correlated with inhibited expression of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 (Ryter *et al.* 2002).

Beneficial effects of CO have been demonstrated in models of ischaemia-reperfusion injury of the heart, kidney, small bowel and liver (Amersi *et al.* 2003; Nakao *et al.* 2003). In a model of lung ischaemia-reperfusion injury, HO-1 knockout mice displayed increased mortality. Inhalation of CO partially compensated for the deficiency and improved survival (Fujita *et al.* 2001).

1.3.4.4.4 Bronchodilatory effects

Carbon monoxide modulates airway responsiveness by acting as a neurotransmitter in parasympathetic ganglia of human and guinea pig airways (Kinhult *et al.* 2001) and by direct effects on smooth muscle (Morita *et al.* 1995). In guinea pigs, inhalation of CO at low concentrations results in bronchodilation via cGMP pathways (Cardell *et al.* 1998b). Carbon monoxide is effective in reducing airway hyperresponsiveness in mice with induced airway inflammation and in normal animals (Ameredes *et al.* 2003). Haem oxygenase activation in ovalbumin sensitised and challenged guinea pigs prevented airway

hyperresponsiveness (Amolki et al, 2004) which the authors postulated could be secondary to a reduction in inflammation or a direct effect on smooth muscle.

1.3.4.4.5 Anti-apoptotic effects

In organ transplantation, exposure to CO has been shown to have anti-apoptotic effects both *in vitro* and *in vivo. In vitro,* exogenous administration of CO or the overexpression of HO-1 prevented TNFα-induced apoptosis of murine fibroblasts by stimulating the p38MAPK pathway (Brouard *et al.* 2000; Petrache *et al.* 2000) and co-operated with NF-κB dependent anti-apoptotic genes to protect against TNFα-mediated endothelial cell apoptosis (Brouard *et al.* 2000). Carbon monoxide exogenously applied at low concentrations inhibited ischaemia-reperfusion induced apoptosis in endothelial cell and smooth muscle cells (Liu *et al.* 2003; Zhang *et al.* 2003). Anti-apoptotic effects may be modulated by different transduction pathways depending upon cell type.

1.3.4.4.6 Anti-proliferative effects

Carbon monoxide has been shown to block cell proliferation of a number of cell types including neoplastic cells, T-cells and vascular smooth muscle cells (Morita *et al.* 1995; Song *et al.* 2002) but conversely has been shown to induce proliferation in endothelial cells (Jozkowicz *et al.* 2003).

1.3.5 Haem Oxygenase Expression and Lower Airway Inflammation

Evidence for a role of HO-1 activity has been identified in the pathogenesis of numerous pulmonary diseases in man including acute respiratory distress syndrome (ARDS), interstitial pulmonary fibrosis, COPD and asthma. Asthma is a chronic inflammatory airway disease associated with increased ROS and inflammatory mediators, all of which have been demonstrated to induce expression of HO-1 (Barnes 1990). The pathogenesis of RAO has some parallels with the pathogenesis of asthma, and oxidative stress has been identified in horses during exacerbation of RAO (Art *et al.* 1999; Deaton *et al.* 2006; Deaton *et al.* 2004a; Deaton *et al.* 2004b). Haem oxygenase-1 is induced in airways during asthma (Carter *et al.* 2004) and it is possible that it will be similarly induced in the airways of horses with RAO (Wyse *et al.* 2006). In an aeroallergen–induced model of asthma,

exogenous CO ameliorated inflammation; specifically by reducing BALF eosinophilia, reducing the expression of IL-5, PGE_2 and LTB_4 and by reducing MUC5AC gene expression and mucus production (Chapman *et al.* 2001; Almolki *et al.* 2004). Concentrations of exhaled CO have been identified to be associated with the clinical severity of asthma (Zayasu *et al.* 1997; Horvath *et al.* 1998; Yamaya *et al.* 2001). In addition, a positive association between concentrations of exhaled CO and stabling has been demonstrated in horses with RAO (Wyse *et al.* 2005). Increased HO-1 expression has been identified in the sputum of asthmatic patients (Horvath *et al.* 1998). Concentrations of exhaled CO are reduced in patients receiving corticosteroid therapy (Horvath *et al.* 1998) and are significantly related to eosinophil counts in sputum (Zayasu *et al.* 1997).

1.4 Inducible Nitric Oxide Synthase (iNOS) in the Respiratory Tract

Nitric Oxide (NO) was regarded as a noxious environmental pollutant present in cigarette smoke and exhaust fumes until several researchers in the late 1980's demonstrated the physiological importance of the molecule. Endothelial cells had been known to release a labile factor known as endothelium derived relaxing factor (EDRF) that diffused to the adjacent muscle layer resulting in relaxation. Following the discovery that EDRF was NO (Ignarro *et al.* 1987; Palmer *et al.* 1987), the diverse roles of NO and other nitrogen oxides in other body functions emerged. Nitric oxide was found to act as a pulmonary vasodilator (Pepke-Zaba *et al.* 1991) and shortly thereafter a number of research groups discovered exhaled NO concentrations were higher in patients with asthma than in normal subjects (Alving *et al.* 1993; Gaston *et al.* 1994; Kharitonov *et al.* 1994; Persson *et al.* 1994). Further research activity has demonstrated numerous physiological and pathological functions of NO.

1.4.1 The Isoforms of Nitric Oxide Synthase

Nitric oxide is produced by a diverse range of resident and inflammatory cell types within the respiratory tract by oxidation of the amino acid L-arginine (Vaughan *et al.* 2003b). This reaction is catalysed by nitric oxide synthase (NOS) which exists in both constitutive (cNOS) and inducible forms (iNOS; Bult *et al.* 1990). Both forms of NOS are present within the airways (Ricciardolo *et al.* 2004). Constitutive forms have a characteristic tissue expression and may be neural (NOS-1 or nNOS) or endothelial/epithelial (NOS3 or eNOS)

in origin. Constitutive NOS is Ca^{2+}- and calmodulin-dependant and releases small quantities (fmol or pmol) of NO within seconds in response to receptor agonists such as acetylcholine and bradykinin.

1.4.2 Regulation of iNOS Expression

Expression and synthesis of iNOS is regulated at a pre-translational level and can be induced by endogenous factors including pro-inflammatory cytokines TNFα, IFN-γ and interleukin1-β (IL-1 β) or exogenous factors such as bacteria, bacterial toxins (especially lipopolysaccharide), viruses, allergens and environmental pollutants (Morris and Billiar 1994; Yan *et al.* 1995; Yeadon and Price 1995; Goldman *et al.* 1996). By contrast, transforming growth factor-β (TGF-β) suppresses expression of iNOS in macrophages (Morris *et al*. 1994). Exogenous corticosteroids also reduce expression of iNOS (Haddad *et al.* 1995). In addition, hypoxia, oxidative injury and tumour cells may stimulate iNOS expression (Yan *et al.* 1995; Yeadon and Price 1995; Goldman *et al.* 1996). By contrast to cNOS, iNOS releases large quantities (nmol) of NO in a sustained manner which may last for hours or days (Ricciardolo *et al.* 2004).

1.4.3 Expression of NOS in the Lower Respiratory Tract

In humans eNOS is constitutively expressed in bronchial epithelium (Shaul *et al.* 1994), type II alveolar epithelial cells (Pechkovsky *et al.* 2002) and the basement membrane of ciliary microtubules (Xue *et al.* 1996). In both humans and animals, nNOS is localised in nerve fibres of airway smooth muscle, however substantial species differences exist (Ricciardolo *et al.* 2004). Nitric oxide is a major mediator of smooth muscle relaxation (Li and Rand 1991; Belvisi *et al.* 1992). Density of nerve fibres decreases from trachea to bronchi (Fischer and Hoffmann 1996) with an associated reduction in neural bronchodilation (Ellis and Undem 1992; Ward *et al.* 1995) mediated by the inhibitory nonadrenergic noncholinergic (iNANC) system (Widdicombe 1998). Similarly, iNANC fibres, mediated by NO, decreased in number from the trachea to the terminal bronchi in equine airways (Yu *et al.* 1994). In human airways, NOS-containing fibres have been identified around submucosal glands but their importance is uncertain (Fischer and Hoffmann 1996). Within the lamina propria, NO is a potent vasodilator and important regulator of plasma extravasation (Erjefalt *et al.* 1994). Nitric oxide synthase immunoreactive neurons are also present within the vagal sensory and sympathetic ganglia (Fischer *et al.* 1996) and within the central nervous system (Forstermann *et al.* 1998).

Neuronal NOS has been identified in non-neural tissues of laboratory animals, namely within the cytoplasm of the respiratory epithelium and vascular endothelium (Ellis and Undem 1992).

Inducible NOS was first identified in macrophages (Pechkovsky *et al.* 2002), however it was soon realised that it was present within the cytosol of a diverse range of cell types (Fischer *et al.* 2002). To date, iNOS has been reported in alveolar type II epithelial cells (Warner *et al.* 1995), lung fibroblasts (Romanska *et al.* 2002), airway and vascular smooth muscle cells (Thomae *et al.* 1993; Xue *et al.* 1994; Griffiths *et al.* 1995), bronchial epithelial cells (Adcock *et al.* 1994; Robbins *et al.* 1994; Watkins *et al.* 1997; Pechkovsky *et al.* 2002), mast cells (Gilchrist *et al.* 2002), endothelial cells (Ermert *et al.* 2002), neutrophils (Boucher *et al.* 1999) and chondrocytes (Kobzik *et al.* 1993; Ricciardolo *et al.* 2006). Under healthy conditions iNOS expression may not be detected (Buttery *et al.* 1994).

1.4.4 Functions of NO within the Lower Respiratory Tract

Nitric oxide has a half life of only 1-5 seconds which is of importance given it possesses an unpaired electron and therefore reacts readily with a range of other molecules such as oxygen, superoxide radicals and transition metals. Nitric oxide may be bioactivated as nitroxyl (NO^-) or nitrosonium (NO^+). The activities of NO are commonly categorised as cyclic GMP-dependant or independent, however with improved understanding of the actions of NO, it is becoming apparent that many of the actions of NO occur via cGMP-dependant and independent mechanisms.

Nitric oxide is a ubiquitous messenger molecule that readily diffuses across cell membranes and the cytoplasm to reach its target receptors alleviating the need for extracellular receptors. Binding to iron within the haem component of soluble guanyl cyclase stimulates conversion of GTP to cGMP and generation of cGMP-mediated effects. Cyclic GMP initiates airway smooth muscle relaxation by reducing the concentration of intracellular Ca^{2+} via stimulation of activated K^+ channels, inhibiting membrane Ca^{2+} channels, activating the Ca^{2+}/ATPase pump in the cell membrane and sarcoplasmic reticulum and inhibiting inositol triphosphate generation (Carvajal *et al.* 2000). In addition, cGMP reduces sensitivity of smooth muscle cells to intracellular Ca^{2+} by stimulation of myosin light chain phosphatase activity (Sauzeau *et al.* 2000).

Nitric oxide released from iNOS acts as an immune effector molecule in the killing of tumour cells (Hibbs *et al.* 1988), halting of viral replication (Karupiah *et al.* 1993) and elimination of bacterial, parasitic and fungal, pathogens (Denis 1991). These actions may be accomplished by deamination of pathogen DNA or *S*-Nitrosylation of cysteine proteases which are critical for virulence and replication of viruses, bacteria and parasites (Saura *et al.* 1999).

Products of NOS activation such as *S*-nitrosothiols act as signalling molecules within the lung, resulting in relaxation of pulmonary vascular smooth muscle and increased minute ventilation in response to hypoxia (Lipton *et al.* 2001). *S*-Nitrosothiols are present in the airways of normal humans in concentrations sufficient to influence airway tone and are decreased in the lungs of patients with asthma (Vaughan *et al.* 2003b). *S*-Nitrosothiol induced bronchodilation is cGMP-independent and mediated by reversible oxidation of thiols on proteins integral to muscular contraction.

1.4.4.1 NO and Bronchodilation

Bronchomotor tone is under the control of cholinergic and adrenergic systems in combination with excitatory and inhibitory non-adrenergic, non-cholinergic mechanisms (eNANC and iNANC). The iNANC represents a parasympathetic pathway distinct from the cholinergic pathway. Nitric oxide is responsible for mediating approximately half of the iNANC response in guinea pig and human airways (Kobzik *et al.* 1993). In humans, the iNANC response in central and peripheral airways is mediated solely by NO (Belvisi *et al.* 1992; Ellis and Undem 1992). Inhibition of NOS potentiates cholinergic neural bronchoconstriction via functional antagonism at a postjunctional level. The apparent importance of the iNANC in human airways has led to the hypothesis that impairment of iNANC function in inflammatory disease states may result in bronchoconstriction. In cystic fibrosis (a condition characterised by neutrophilic airway inflammation) and asthma, NO-induced bronchodilation is reduced (Mackay *et al.* 1991; Barnes and Liew 1995; Miura *et al.* 1997). In addition, NO has been demonstrated to have a role in the regulation of motility of the equine jejunum and ventral colon (Rakestraw *et al.* 1996; Van Hoogmoed *et al.* 2000).

A number of studies have demonstrated that nitrovasodilators reduce bronchoconstriction both at baseline and following bronchoconstriction induced to mimic disease states (Aviado *et al.* 1968; Dupuy *et al.* 1992; Gruetter *et al.* 1989; Hogman *et al.* 1993). However, effects on pulmonary compliance are minimal, suggesting a greater effect on the

larger airways (Hogman *et al.* 1993) that is consistent with the distribution of the distribution of iNANC fibres. Following allergen or viral challenge, eNOS activity is transiently (4-6 hours) reduced, resulting in airway hyperresponsiveness and bronchoconstriction possibly as a result of limited availability of substrate (de Boer *et al.* 2001). A subsequent increase in iNOS expression results in attenuated airway hyperresponsiveness, reduced bronchoconstriction and a detectable increase in NO metabolites in BALF (Liu *et al.* 1993; Schuiling *et al.* 1998; Toward and Broadley 2000; Samb *et al.* 2001; Hjoberg *et al.* 2004). Inhibition of NOS (Filep *et al.* 1993; Figini *et al.* 1996a; Figini *et al.* 1996b; Ninomiya *et al.* 1996; Ali *et al.* 1997; Emanueli *et al.* 1998) or removal of the airway epithelium (Folkerts and Nijkamp 1998) results in smooth muscle contraction and bronchoconstriction. Airway epithelium is therefore central to maintenance of airway reactivity and is not merely a protective barrier.

1.4.4.2 NO and Circulation within the Lower Respiratory Tract

Nitric oxide is released from the endothelium of the pulmonary vasculature (Ignarro 1989; Persson *et al.* 1990). Release of NO in the pulmonary circulation is reduced in chronic hypoxia (Adnot *et al.* 1991) and has been identified in human patients with COPD and cystic fibrosis (Dinh-Xuan *et al.* 1991). Endogenous and exogenous NO have a significant influence on bronchial vasodilation (Sasaki *et al.* 1995; Charan *et al.* 1997). Endogenous NO has been shown to have a role in the regulation of plasma extravasation in response to certain allergens (Miura *et al.* 1996), inflammatory mediators (Kageyama *et al.* 1997) and lipopolysaccharide (Bernareggi *et al.* 1997). However, alteration of blood flow as a result of NO release may be a confounding factor in the assessment of plasma extravasation.

1.4.4.3 NO and Airway Secretion

Airway secretion in response to inflammatory mediators such as histamine, TNF-α, methacholine and bradykinin is mediated by a mechanism involving NO signalling (Adler *et al.* 1995). Furthermore, administration of the NO generator isosorbide dinitrate significantly increases mucosal gland secretion (Nagaki *et al.* 1995).

In bovines, ciliary motility is enhanced by iNOS induction (Jain *et al.* 1995). Ion channels within the airways are modulated by NO which may have implications for the constituents of epithelial lining fluid (Duszyk and Radomski 2000).

1.4.4.4 NO and Oxidative Stress: "Nitrosative Stress"

Nitric oxide radicals are produced in a range of cell types including nerves, macrophages, fibroblasts, airway and vascular smooth muscle cells. Enzyme systems involving xanthine oxidase, *P*-450 monooxygenase, cyclooxygenase, lipoxygenase, indole amine dioxygenase and monoamine oxidase produce large quantities of ROS (Fridovich 1984; Vallyathan and Shi 1997). Inhaled noxious substances such as ozone, nitrogen dioxide, cigarette smoke and dust are potent stimuli for the release of RNS and deplete anti-oxidants within the epithelial lining fluid (Vallyathan and Shi 1997; Krishna *et al.* 1998).

Measuring nitrosative stress can be challenging as the fate of reactive nitrogen species is often unclear, metabolites are unstable and their half lives are short. Some products, for example nitrate and nitrite, are more stable and can be measured in plasma (Kelm 1999); however levels of both these products are influenced by dietary intake, reducing their diagnostic value. Under conditions of low airway pH, for example asthma, nitrite may be converted back into NO (Hunt *et al.* 2000). Increased NO production in the airways and release in breath may therefore represent nitrite conversion as well as iNOS activity.

Immunohistochemistry has been used to identify 3-nitrotyrosine residues within tissue samples (Saleh *et al.* 1998) and biological fluids (Ohshima *et al.* 1999). 3-Nitrotyrosine is formed readily from hydrogen peroxide and nitrite in a process mediated by myeloperoxidase and eosinophil peroxidise (Kettle *et al.* 1997; Eiserich *et al.* 1998; Wu *et al.* 1999). 3-Nitrotyrosine contributes to cellular dysfunction and is a specific biomarker of nitrosative stress.

Reactive nitrogen species have an array of potential targets; some of the most biologically important are tyrosine residues, thiols and haem groups within proteins (Fang 1997; Gaston 1999; van der Vliet *et al.* 1999). Incorporation of the NO_2 group alters the stearic and electronic properties of proteins affecting their ability to act as enzymes and structural molecules. Protein binding can be reversible and therefore a potential signalling event (Kamisaki *et al.* 1998). Reactive nitrogen species alter lipid oxidation pathways (Rana *et al.* 2001), damage DNA (Zingarelli *et al.* 1996) and inhibit mitochondrial respiration (Packer and Murphy 1995) by mechanisms that remain to be elucidated. The end result of these effects may be apoptosis or necrosis, dependent upon the severity of damage (Murphy 1999). The effects of reactive nitrogen species in both host and pathogen may be mediated by the mitogen-activated protein kinase (MAPK) pathway (Nabeyrat *et al.* 2003).

Peroxynitrite increases airway hyper-responsiveness, respiratory epithelial damage and eosinophil activation in guinea pigs (Sadeghi-Hashjin *et al.* 1996). In addition, it inactivates surfactant (Haddad *et al.* 1994) and inhibits protein phosphorylation by tyrosine kinases interfering with signal transduction mechanisms (Gow *et al.* 1997). Peroxynitrite may also contribute to airway inflammation by activating matrix metalloproteinases (Okamoto *et al.* 1997), inactivating α1-antiprotease (Whiteman *et al.* 1999) and enhancing production of the neutrophil chemoattractant IL-8 (Filep *et al.* 1998). Peroxynitrite has effects on the function of eNOS with the net result being increased release of NO and enhanced release of O_2^- amplifying oxidative stress (Zou *et al.* 2002).

Whilst pro-oxidants are generally considered detrimental, reduction in reactive nitrogen species may result in reduced defence against invading pathogens and compromised function of NO as a paracrine messenger (Swislocki *et al.* 1995). Generalised inhibition of NOS is likely to result in deleterious effects; however targeted inhibition of NADPH oxidase and reduced production of super oxide (the precursor for production of many reactive nitrogen species) may have significant beneficial effects (Ricciardolo *et al.* 2006).

1.4.4.5. NO and Immune-Inflammatory Responses

Production of NO is increased in response to inflammatory cytokine production. In laboratory animals, macrophages have been shown to produce nitrite in a dose- and time-dependent manner following activation with lipopolysaccharide and IFN-γ, a process that is blocked by glucocorticoids (Jorens *et al.* 1991). Granulocyte-macrophage colony stimulating factor (GM-CSF) and muramyl dipeptide, a bacterial cell wall component, enhance IFN-γ-induced nitrite production (Jorens *et al.* 1993). Other cytokines, including TGF-β, IL-4 and IL-10 inhibit the expression of iNOS (Nijkamp and Folkerts 1994). In addition, NO reduces the production the inflammatory cytokines TNF, IL-1, MIP-1a from human macrophages during inflammatory conditions, suggesting a modulatory role in disease (Thomassen *et al.* 1997). Nitric oxide also suppresses antigen-induced degranulation, mediator release and cytokine expression by mast cells. *In vivo*, NO inhibits mast cell dependant histamine-induced vasodilation, vasopermeation and leukocyte-endothelial cell attachment (Coleman 2002).

T-helper type 1 cells (in contrast to Th2 cells) can be activated by specific antigens to produce large quantities of NO (Barnes and Liew 1995). Nitric oxide inhibits further interleukin production from Th1 cells whilst perpetuating Th2 cell-mediated inflammation in allergic and non-allergic disease. In models of allergic airway disease, iNOS knockout

mice show reduced eosinophil tracking, plasma extravasation and airway obstruction (Xiong *et al.* 1999). Increases in IFN-γ were identified in the knockout mice; however there was no reduction in IL-4 or Il-5, suggesting that iNOS induction results in perpetuation of antibody-mediated allergic inflammation by downregulation of IFN-γ activity. In addition, IL-4 increases in response to NO (Slater *et al.* 2006). Furthermore, NO inhibits macrophage-derived IL-12 release, reducing induction and amplification of the Th1 response (Huang *et al.* 1998).

Nitric oxide promotes production of chemokines for eosinophils in mice indicating the possibility of a positive-feedback loop with inflammatory cells producing NO that promotes further leukocyte recruitment (Ricciardolo *et al.* 2004).

1.4.4.6 NO and Airway Hyperresponsiveness

Pre-treatment with NOS inhibitors results in potentiation of bradykinin and methacholine-induced airway hyperresponsiveness (AHR) consistent with a protective role for endogenous NO in allergic airway disease (Ricciardolo *et al.* 1996). However, NO synthesis was impaired following AHR induced by bradykinin in patients with severe asthma. The authors proposed that this might be due to a reduction in eNOS in severely asthmatic patients (Ricciardolo *et al.* 1997). Airway hyperresponsiveness to bradykinin due to allergen exposure in asthma is a result of impaired production of NO subsequent to downregulation of eNOS and upregulation of iNOS within the respiratory epithelium (Ricciardolo *et al.* 2001). Polymorphisms of the eNOS gene may be associated with predisposition to asthma in humans (Lee *et al.* 2000; Storm van's Gravesande *et al.* 2003). Restoration of eNOS has been advocated as a potential treatment strategy for asthma (Ricciardolo *et al.* 2001).

Treatment with corticosteroids has been demonstrated to have a protective effect in severely asthmatic patients, presumably as a result of suppression of iNOS and renewed activity of eNOS (Redington 2006).

1.4.4.7. NO and Cell Proliferation/Neoplasia

The effects of nitrogen oxides on cell division, survival and cell death are dependent upon chemical species, redox state, concentration and target type; the results of studies into these effects being complex and paradoxical (Lipton *et al.* 1993). Administration of NO donors has been shown to reduce proliferation of human airway smooth muscle cells (Hamad *et*

al. 1999; Patel *et al.* 1999). In bronchiolitis, NO has been suggested to increase destruction of epithelium and stimulate fibroblast activity (Romanska *et al.* 2000). At physiological concentrations NO appears to protect against apoptosis by augmenting antioxidant defences and inactivating caspases (Hoffmann *et al.* 2001; Haendeler *et al.* 2002).

1.4.5 NO and Respiratory Tract Inflammation

Levels of NO in exhaled breath are determined by a number of factors; NO production by multiple cell types in the airways and parenchyma, diffusion of NO into the capillary circulation, alveolar ventilation and bronchial airflow (Hyde *et al.* 1997). All 3 NOS isoforms contribute to the production of exhaled NO and results of studies performed are conflicting as to which isoform is the most important. In asthma, both iNOS (Saleh *et al.* 1998) and eNOS (Storm van's Gravesande *et al.* 2003) are upregulated with the assumption that iNOS in the epithelium is the main source. However, data from animal models have indicated the importance of cNOS in disease states (De Sanctis *et al.* 1997; Vaughan *et al.* 2003a).

Rate of NO production is dependent upon numerous factors including the presence of prokaryotic denitrifying bacteria within both upper and lower airways (Vaughan *et al.* 2003b), *S*-nitrosothiols, catabolic enzymes (Dweik *et al.* 2001) and effects of enzymatic and other processes that regulate airway pH and nitrite reduction (Hunt *et al.* 2002). In addition, rate of NO production will be influenced by the activity of arginase 2 and metabolic enzymes that regulate the endogenous NOS inhibitor asymmetric dimethyl arginine (Nelin *et al.* 2002).

Nitric oxide concentrations in exhaled breath collected from humans can be measured by chemilluminescence analysers (Nyman *et al.* 1991), however concentrations are highly dependent upon expiratory flow rate (Phillips *et al.* 1996) such that careful standardisation of measurement is required (Kharitonov *et al.* 1997; American Thoracic Society 1999; Baraldi and de Jongste 2002). Patients with asthma exhale greater levels of NO than unaffected subjects (Kharitonov *et al.* 1994; Gratziou *et al.* 1999) and the increase is predominantly of lower airway origin (Massaro *et al.* 1995; Kharitonov *et al.* 1996a). In asthma, exhaled NO has been used to assess disease severity and response to treatment with corticosteroids (Kharitonov *et al.* 1996b; Sippel *et al.* 2000). Corticosteroids have a direct inhibitory effect on iNOS expression in addition to general downregulation of the inflammatory response. Exhaled NO concentrations in asthmatic patients correlated with

AHR (Jatakanon *et al.* 1998) and peak flow variability (Lim *et al.* 1999). In addition, correlation of exhaled NO with eosinophil counts in peripheral blood (Silvestri *et al.* 1999), BALF (Lim *et al.* 1999), saliva and bronchial biopsies has been identified (Payne *et al.* 2001). From the results of these studies it has been suggested that production of endogenous NO may be contributory to eosinophilic inflammation and be deleterious in allergic airway disease. Alternatively, production of NO and eosinophilia may be stimulated by similar pathological processes within disease states. Increased concentrations of exhaled NO have been demonstrated in other respiratory diseases in humans including COPD, rhinitis, bronchiectasis, pulmonary sarcoidosis and active fibrosing alveolitis. Conversely, reduced concentrations of NO have been identified in primary ciliary dyskinesia, cystic fibrosis and pulmonary hypertension (Ricciardolo *et al.* 2004). The reasons for reduced production of NO in these conditions are unknown (Ricciardolo *et al.* 2004).

Therapeutic benefits of inhaled NO in humans with respiratory disease have been investigated. Inhaled NO diffuses into and selectively dilates the pulmonary vasculature of ventilated lung regions resulting in a selective increase in blood flow to better ventilated regions (Fratacci *et al.* 1991; Frostell *et al.* 1991). Nitric oxide rapidly binds to haemoglobin within the circulation preventing systemic effects of inhaled NO in contrast to other vasodilators. These properties have resulted in the use of inhaled NO in the treatment of pulmonary hypertension. Though the potential benefits in respiratory disease are yet to be fully investigated (Bates and Silkoff 2003), NO is used at 5-30ppm in human patients with severe respiratory disease such as ARDS (Zapol and Hurford 1994).

Potentially beneficial effects of NO include neuromodulation by mediation of iNANC nerve activity, smooth muscle relaxation, attenuation of bronchoconstrictor stimuli, downregulation of mast cell and Th1 cell-mediated inflammatory responses and the killing of invading microorganisms.

Potentially deleterious effects of NO and RNS include vasodilation and plasma extravasation, increased airway secretions, impaired ciliary motility, increased Th2-mediated eosinophilic inflammation, necrosis and apoptosis. A selective inhibitor of iNOS [L-N^6-(1-iminoethyl)-lysine] has been shown to reduce exhaled NO in healthy volunteers and asthmatics. In rodent models of allergic airway inflammation iNOS inhibitors have been shown to decrease eosinophil infiltration and cytokine release. Some authors have

suggested that iNOS inhibitors may have a therapeutic benefit in cases of allergic airway disease that are refractory to corticosteroid treatment (Hansel *et al.* 2003).

1.4.6 NO in Equine Health and Disease

Little research has been performed into the expression of NOS and synthesis of NO in the horse. Increased expression of iNOS has been identified in leukocytes within the mucosa and submucosa of the intestine and vasculature of horses with ischaemic intestinal injury (Mirza *et al.* 1999; Mirza *et al.* 2005). Plasma and peritoneal fluid NO concentrations were increased in horses with strangulating lesions of the small intestine but not in those with colon volvulus (Mirza *et al.* 1999; Mirza *et al.* 2005); the reasons for this discrepancy are uncertain. Increased nitrotyrosine staining was also identified, consistent with the presence of peroxynitrite secondary to increased NO and superoxide production (Mirza *et al.* 1999; Mirza *et al.* 2005). Evidence of NO synthesis within the endothelium has also been identified in studies performed to enhance understanding of the pathogenesis of laminitis (Cogswell *et al.* 1995; Moore *et al.* 2005). Infusion of lipopolysaccharide and administration of *Strongylus vulgaris* larvae both failed to induce an increase in plasma NO despite an increases in serum TNF and IL-6 concentrations (Bueno *et al.* 1999; Hubert *et al.* 2004). Proposed explanations for the absence of a detectable increase in NO concentration in these horses were an insufficient inflammatory stimulus, insufficient cytokine expression, sample degradation prior to analysis and a different mechanism and time course of iNOS expression than seen in other species (Hubert *et al.* 2004).

Nitric Oxide concentration has been shown to increase from 1ppb at rest to 7ppb in exercising horses (Mills *et al.* 1996a) and the administration of inhaled NO reduced pulmonary arterial pressure (Mills *et al.* 1996b). Inhibition of NOS in exercising horses resulted in an increase in plasma lactate in one study, suggested to be a result of changes in skeletal blood flow and/or metabolic activity (Mills *et al.* 1999). In addition, NO was shown to be important in thermoregulation during exercise (Mills *et al.* 1997).

To the author's knowledge investigation of NOS expression and NO production in equine respiratory disease is limited to the findings of a single study in which 7 horses with SPARAO and 6 normal horses were compared (Costa *et al.* 2001). Nitric oxide concentrations in plasma, BALF and ELF were determined using a chemilluminescence method whilst iNOS, nitrotyrosine and nicotinamide (NT) adenine dinucleotide phosphate diaphorase (NADPHd) were localised in formalin-fixed lung specimens using

immunohistochemical and histochemical staining. Plasma concentration of NO in affected horses was non-significantly increased over non-affected controls. Nitric oxide concentrations in BALF and ELF were not different between groups. Immunoreactivity of iNOS was increased in bronchial epithelial cells in 3 of 5 lung lobes. Staining for NT and NADPHd did not differ between the two groups. Staining of peribronchiolar leukocytes for iNOS, NT and NADPHd activity was also assessed and did not differ significantly between groups. Statistical models showed that plasma NO concentrations could best be modelled by 3 variables; percentage of mast cells in BALF, percentage of lymphocytes in BALF and changes in pleural pressure (Costa *et al.* 2000b).

The absence of a detectable increase in NO production in the lower respiratory tract in the study by Costa *et al.* (2000) was unexpected given an increase in exhaled NO has been reported in multiple studies of human patients suffering from asthma (Alving *et al.* 1993; Kharitonov *et al.* 1994; Massaro *et al.* 1995; Kharitonov *et al.* 1996a). The effects of inhaled NO at a concentration of 5ppm on pulmonary mechanics in horses with histamine-induced bronchoconstriction have been investigated (Sweeney *et al.* 1999). Nitric oxide at this concentration (approximately 1000x higher than that reported for normal resting horses) partially reversed the changes in dynamic compliance and airway resistance induced by histamine. However, effects were short-lived and changes in pulmonary function were only partially reversed. This was not unexpected as in addition to bronchoconstriction, histamine induces mucosal oedema and increased airway secretions.

One of the aims of the current study was to further investigate the production of NO in the lower airways of horses with respiratory disease; specifically to characterise the investigation of iNOS in leukocytes of BALF.

1.4.7 Interactions of NO and HO-1

As understanding of the HO and NOS enzymes increases, it is increasingly clear that there are significant, and complex, interactions between the two systems (Ryter and Otterbein 2004). Understanding of these interactions remains limited; however some of the suggested mechanisms are listed below:

1.4.7.1 HO Regulation of NOS

Induction of HO-1 may inhibit production of NO in a number of ways (Maines 1997):

1. NO is a haemoprotein. Activation of HO-1 and reduction in available haem may result in decreased synthesis of NOS.
2. Induction of HO-1 may result in increased turnover of NOS. Increased concentration of HO-1 has been associated with decreased P450.
3. CO is known to bind to NOS and may result in deactivation of the enzyme.
4. Iron may inhibit NOS transcription.
5. Both systems require NADPH and electrons therefore competition may exist. HO-1 is likely to function at the expense of NOS.

By contrast, NO and peroxynitrite have been demonstrated to up-regulate HO-1 expression (Foresti *et al.* 1999; Foresti and Motterlini 1999; Liang *et al.* 2000) and it has been suggested that CO may assume many of the roles of NO under oxidative conditions (Foresti and Motterlini 1999). In addition, CO has been shown to induce iNOS in hepatocytes which in turn induces HO-1 (Zuckerbraun *et al.* 2003). The authors of this study suggested that the effect may be tissue specific as CO was associated with inhibition of NOS in other models of tissue injury (Nakao *et al.* 2003).

1.4.7.2 NOS regulation of HO-1

Nitric oxide has been shown both to inhibit (Willis 1995; Wang *et al.* 2003) and activate (Motterlini *et al.* 1996) HO activity. Nitric oxide has been suggested to be the strongest inducer of HO-1 (Motterlini *et al.* 1996). It is therefore possible that many of the effects of NO may be modulated via HO-1 (Ryter and Otterbein 2004). Nitric oxide may also induce HO-1 activity by modulating the activity of enzymes responsible for regulating levels of free iron and the synthesis of haem and ferritin.

Inhibitory activity may be due to NO orchestrated damage (due to free radical properties) to the proteins of haem-oxygenase and its substrate haem. Nitric oxide has a higher affinity for haem than that demonstrated by oxygen and the presence of NO prevents oxidation (Ryter and Otterbein 2004).

It has been suggested that NOS and NO will have varied effects according to the type of HO isoform and tissue type.

1.5 Evaluation of Inflammation in the Equine Lower Respiratory Tract

1.5.1 History, Clinical Signs and Scoring Systems

The history of disease and timing of clinical signs are often strongly suggestive of the type of respiratory disease present. Recurrent airway obstruction and SPARAO are both seasonal disorders; RAO typically is associated with periods of winter housing and SPARAO is associated with warmer drier weather when horses are at pasture. However, it is likely that there is a spectrum of disease with considerable cross-over between the conditions. For example, it has been reported that 10% of horses with RAO also suffer from SPARAO and approximately 50% of horses with SPARAO demonstrate hypersensitivity to hay and straw (Mair 1996). Both conditions occur in horses over 5 years of age at a median of 12 years (Aviza *et al.* 2001). All breeds may be affected (McPherson *et al.* 1979a; Couetil and Ward 2003). History is important in differentiating RAO from IAD; the latter typically affects younger horses in training and is not seasonal or recurrent in nature (Couëtil *et al.* 2007).

Horses with RAO may present with a range of clinical signs according to disease severity. The typical clinical signs of RAO are coughing and nasal discharge which occur in up to 84% and 54% of clinical cases respectively (Dixon *et al.* 1995b). Respiratory rate may be normal or increased, whilst temperature, heart rate and general attitude are normal in all but severe acute disease. Respiratory distress, characterised by a biphasic forced abdominal effort accompanied by forced expiration, nostril flaring and an outstretched neck may be apparent in acute exacerbations of disease (Couetil and Hinchcliff 2004; Lavoie 2007). With chronicity, hypertrophy of the external abdominal oblique muscles may result in a visible “heave line”. Recurrent airway obstruction may also be subclinical with the only presenting complaint being a reduction in performance. A tentative diagnosis in acute cases can usually be made easily from the clinical signs and history. Auscultation is insensitive and findings may be abnormal in less than 50% of horses with RAO (Dixon *et al.* 1995b); however expiratory wheezes indicative of intra-thoracic airway obstruction and crackles indicative of fluid within the airways may be audible. The sensitivity of auscultation may be increased by increasing the depth of breathing by applying a “re-breathing bag” over the nostrils and in so doing limiting the supply of oxygen, increasing inspired carbon dioxide and inducing hypercapnia (Roy and Lavoie 2003). In less severe and subclinical cases, diagnosis may require further diagnostic modalities and

differentiation from IAD may be challenging. A comparison of the typical features of RAO and IAD are presented in Table 1.

Table 1.5.1 Comparison of clinical findings of RAO and IAD. Adapted from Couetil and Hinchcliff (2004).

	RAO	IAD
Age	>5 years	> 1 year
Activity Level	any	moderate/high
Duration	months/years recurrent	1-6 months not recurrent
Cough	chronic intermittent	1/3 cases
Exudate	moderate/marked mucopurulent	mild/moderate mucoid
Lung sounds	Increased breath sounds, wheezes, crackles	normal
Increased respiratory effort	often evident	sometimes evident, less severe
Exercise intolerance	moderate/marked	mild/moderate
BALF cytology	Neutrophilia >25%	Neutrophilia >5% Eosinophilia >1% Mastocytosis >2%

Clinical scoring systems have been developed to allow comparison of airway obstruction between groups, primarily for research purposes. Grades are assigned to the common clinical signs such as cough, nasal flaring, abdominal excursions and movement of the anus. Simple scoring systems grading nasal flaring and abdominal effort have been shown to correlate with more objective measures of pulmonary function (Rush *et al.* 1998; Costa *et al.* 2000b; Robinson *et al.* 2000).

As the clinical signs of RAO are often apparent to owners, questionnaire methods have been used in the diagnosis of RAO for the purposes of epidemiological studies and also in the investigation of risk factors for development of disease. A risk-screening questionnaire (RSQ) for RAO was constructed and validated in order to investigate RAO in Great Britain (Hotchkiss *et al.* 2006; Hotchkiss *et al.* 2007). The questions included in the RSQ were derived from a review of the scientific literature, survey of equine practitioners in the UK and consultation with 19 experts using a modified Delphi technique. The RSQ was validated against a reference standard of a veterinary diagnosis including respiratory cytology. A receiver-operating characteristic (ROC) curve was used to select a positive cut-off of 0.87 for the RSQ for RAO. This suggested that the RSQ had a sensitivity of 0.83 (95% confidence interval=0.59-0.96) and specificity of 0.85 (0.74-0.93) for the diagnosis of apparent RAO (compared to all other diagnoses) (Hotchkiss *et al.* 2006). A horse owner assessed respiratory signs index (HOARSI) was developed by one group to determine the

severity of clinical signs in a related population known to have a high prevalence of RAO (Jost *et al.* 2007; Ramseyer *et al.* 2007).

1.5.2 Endoscopy

Endoscopic maps of the equine bronchial tree have been reported (Smith *et al.* 1994); however clinical assessment is generally limited to the trachea by the small size of the bronchi relative to the endoscopes used in practice. Endoscopy of the lower airways in horses with RAO generally reveals mucopus of varying quantities within the trachea. The presence of mucus is a characteristic sign of RAO (Robinson *et al.* 1996) but is not specific for the disease (Dixon *et al.* 1995b). There may be considerable overlap between the degree of mucus accumulation in healthy horses, stabled horses, horses with IAD and horses with RAO. Whilst the presence of isolated droplets of mucus may be considered normal, rarely do horses with RAO have no or only a few droplets of mucus (Gerber 2001). Mucus accumulation has been found to be associated with coughing (Dixon *et al.* 1995c; Christley *et al.* 2001; Robinson *et al.* 2003). Further, mucus scores increase in horses with RAO (but not in healthy horses), with stabling and feeding of hay (Gerber *et al.* 2004a). A number of authors have developed systems for grading mucus accumulation (Burrell 1985; Dixon *et al.* 1995c; Gerber *et al.* 2001; Gerber *et al.* 2003a; Gerber *et al.* 2004a) but the best validated and now most widely accepted is that reported by Gerber *et al*. (2004). In that study mucus grading correlated with neutrophilic airway inflammation as defined by cytology of bronchoalveolar lavage fluid. The significance of mucus within the airways is undetermined, however an association between mucus scores of grade 2 or higher and reduced racing performance has been demonstrated (Holcombe *et al.* 2006).

Endoscopic evidence of oedema and blunting of the carina has been described as a measure of pulmonary disease (Dixon *et al.* 1995c; Mair 1996) and the presence of distended and irregular bronchial walls thought to be suggestive of bronchiectasis (Lavoie *et al.* 2004). Airway erythema may also be evident (Dixon *et al.* 1995c). However, a recent study cast doubt on the value of assessing the tracheal septum by failing to demonstrate correlation with mucus accumulation and airway neutrophilia (Koch *et al.* 2007). Although endoscopic findings are frequently indicative of airway inflammation, findings rarely allow diagnosis of specific diseases: for further characterisation collection of airway secretions is generally required.

1.5.3 Cytological Examination of Tracheo-Bronchial Secretions

Cytological examination of airway secretions is arguably the most useful single diagnostic test for differentiating lower airway diseases. The timing of sampling is important as increases in inflammatory cells and bacteria may be identified within 6 hours of transport, tying up or head elevation (Raidal *et al.* 1995). In such situations, provided the horse is not dehydrated, mucociliary clearance should ensure a return of airway secretions to normal within 12 hours. Exercise may result in an increase in secretions that are probably more representative of the lower airways than samples collected prior to exercise (Martin *et al.* 1999; Malikides 2004; Malikides *et al.* 2007). In comparison to relative percentages of neutrophils in pre-exercise samples, higher neutrophil percentages have been identified after exercise in TAs (Malikides *et al.* 2003) but not in BALF (Clark *et al.* 1995).

1.5.3.1 Tracheal aspirates

Tracheal aspirates may be obtained by a number of techniques, each having advantages and disadvantages. Transtracheal aspiration was adapted from a human technique prior to the availability of endoscopy (Mansmann and Knight 1972). This technique is still relevant as it is less likely to result in contamination of the sample than endoscopic techniques and is performed when infectious disease is suspected and contamination by oropharyngeal flora is undesirable. The major disadvantages of the transtracheal technique are inability to visualise the airways (allowing assessment of pathology and aiding collection of the sample) and the potential for cellulitis/chondritis at the entry site (Mair 1987). Fibreoptic and videoendoscopic techniques are less invasive, associated with fewer complications and preferred by most clinicians. However, endoscopic techniques inevitably result in contamination of the sample by oropharyngeal secretions unless guarded and plugged catheter systems are used in the hands of an experienced clinician (Sweeney *et al.* 1989; Darien *et al.* 1990). Plugged guarded catheters were shown to be effective in preventing bacterial contamination in 15 of 18 samples collected in one study (Christley *et al.* 1999). Risk of contamination increases if the horse is refractory to the procedure or coughs frequently. If the operator is inexperienced contact may be more likely to occur between the contaminated endoscope and the sample. Endoscopic techniques are preferred for the investigation of non-infectious lower airway diseases as visualisation of the airways is advantageous.

Neutrophil percentages in TAs have been shown to correlate with signs of respiratory disease, the presence of bacteria, the presence of mucus and the presence of an increased leucocyte count (Chapman *et al.* 2000; Christley *et al.* 2001; Gerber *et al.* 2004a). Whilst neutrophilia is consistent with respiratory disease of either infectious or non-infectious origin, interpretation is complicated by the wide range of results found in normal horses (3-83%) (Larson and Busch 1985; Derksen *et al.* 1989) and in horses with RAO (7-96%) (Mair *et al.* 1987; Winder *et al.* 1990; Dixon *et al.* 1995c). In RAO, correlation with TA cytology results and lung histology is poor and the validity of the technique for diagnosing RAO has been questioned (Larson and Busch 1985; Derksen *et al.* 1989; Winder *et al.* 1990; Traub-Dargatz *et al.* 1992). The major advantage of TA over BAL is the collection of samples for bacterial culture (Rush and Mair 2004).

1.5.3.2 Bronchoalveolar Lavage (BAL)

Bronchoalveolar lavage was adapted for use in the horse following successful use in human medicine (Viel 1983) and was subsequently validated for the detection of lower airway inflammatory changes in horses (Derksen *et al.* 1985b; Derksen *et al.* 1989; Fogarty and Buckley 1991; Sweeney *et al.* 1992a).

Initially the procedure was performed under general anaesthesia (Viel 1983) using fibreoptic endoscopes. The methods of BAL have since been modified such it can be performed under standing sedation. Sedation with α-2 agonists combined with butorphanol are recommended as they have an anti-tussive effect and reduce discomfort experienced by some horses. Coughing may still be elicited by contact with the larynx, carina or major bronchi resulting in distress to the horse and added difficulty in obtaining a sample: in such cases lignocaine may be instilled into the trachea. Both lignocaine and butorphanol have been demonstrated to reduce coughing prior to BAL (Westermann *et al.* 2005).

Endoscopes must be at least 1.5m in length in order to reach the lower airways and an external diameter of 8 mm is desirable to permit wedging of the endoscope in a 4th generation bronchus (Sweeney and Beech 1991). Development of a BAL technique utilising sterile siliconised polypropylene tubing is an alternative to the use of fibreoptic endoscopes (Fogarty 1990a, 1990b). The structure of the bronchial tree is such that in most cases the tube passed into the right or left caudodorsal lobe. Samples obtained showed good qualitative correlation with pathological changes (Fogarty 1990b). Commercially available BAL tubes measure 2.5 - 3m in length, 10mm in external diameter and are cuffed at the distal end allowing an improved seal to that which can be achieved with an

endoscope. Most clinicians find that the improved seal makes collection of a diagnostic sample easier than with an endoscope, however it is advantageous to visualise the airways prior to BAL and endoscopic collection may be preferable if a sample from a specific bronchus is required. Recurrent airway obstruction diffusely affects the lower airways so lavage of specific airways is not generally indicated. In IAD changes are generally considered to be diffuse and BAL is preferred for diagnosis (Couëtil *et al.* 2007).

There is no standardised protocol for performing BAL and variation in BALF collection methods may influence the results of subsequent BALF analysis. Fogarty (1990a) showed that when BAL was performed, an area of lung surface measuring 12cm in diameter was infused which represented only a fraction of the total lung volume. Further investigations have demonstrated cytological findings of a BALF sample are comparable regardless of the region from which samples are collected (Sweeney *et al.* 1992b; McGorum *et al.* 1993e) and it is accepted that the site of BAL is not critical when investigating diffuse diseases of the peripheral lung such as RAO (McGorum *et al.* 1993e; Jean 1996). Volumes of saline used for the procedure have varied from 50 to 500 ml with a greater percentage of neutrophils and fewer mast cells being recovered when smaller volumes are used, presumably representative of a bronchial lavage (Sweeney *et al.* 1992b). It was suggested that a volume in excess of 50ml should be used to ensure lavage of the alveoli (Sweeney and Beech 1991). Volumes of 300ml have been generally adopted although some clinicians prefer to perform 3 sequential 100ml aliquots rather than a single 300 ml lavage. Whilst Mair *et al.* (1987) found no difference in these 2 methods, Sweeney *et al.* (1992) showed that sequential lavages increased the recovery of BALF and increased the proportion of macrophages. Pickles *et al.* (2002a) found that when sequential aliquots of BALF were compared, there were no clinically relevant differences indicating that all aliquots may be considered equal and even small volumes of recovered fluid are of diagnostic value (Pickles *et al.* 2002a). In a research setting, the effects of BAL on pulmonary function tests needs to be considered (Leguillette and Lavoie 2006). Bronchoalveolar lavage induces a localised influx of neutrophils into the lavaged portion of the lung, resulting in neutrophilia of subsequent lavage fluids for at least 48 hours (Sweeney *et al.* 1994).

Bronchoalveolar lavage is not considered a suitable technique for the investigation of infectious lower airway disease. Bacterial pneumonia generally affects the cranioventral lung lobes which are not sampled if BAL tubes are passed blind. Transendoscopic methods may be used to sample the cranioventral lobes but these samples rarely yield representative

bacterial cultures (Sweeney *et al.* 1991). Furthermore, sample contamination with orophayngeal flora inevitably occurs as a result of the tube passing via the oropharynx unguarded.

Discrepancies may be observed between the results of TA and BAL cytology in horses (Derksen *et al.* 1989; Traub -Dargatz *et al.* 1992; Hughes *et al.* 2003; Malikides *et al.* 2003). However, other investigators have found close correlation between the results of TA and BAL cytology (Dixon *et al.* 1995c). The reasons for these discrepancies are not understood but may indicate that 1) nucleated cell populations vary between different segments of the airways and 2) in IAD, disease may be restricted to certain regions of the lower airways. Tracheal aspirates contain large quantities of mucin and large numbers of degenerate cells. It has been proposed therefore that BALF is a more reliable indicator of distal airway disease because cells are better preserved, there is less variability in normal cell populations and there is greater correlation with the pathological changes observed at histopathology (Larson and Busch 1985; Derksen *et al.* 1989; Dixon *et al.* 1995c). As RAO is a disease of the distal airways it is accepted that BAL is the gold-standard for diagnosis (Robinson 2001). There is less agreement with respect to the diagnosis of IAD. The published consensus view is that BAL is required to confirm the diagnosis (Couëtil *et al.* 2007). This view is not universally accepted as there is a sub-set of horses, especially young racehorses, that show marked changes on TA cytology without alterations in BALF cytology (Derksen *et al.* 1989; Traub-Dargatz *et al.* 1992; Hughes *et al.* 2003; Malikides *et al.* 2003). The results of TA are used widely in the investigation of IAD in young racehorses (Burrell *et al.* 1996; Chapman *et al.* 2000; Christley *et al.* 2001). In older horses a high proportion of neutrophils may be present in TA samples in the absence of clinical evidence of disease (Robinson *et al.* 2006) and hence the relevance of TA cytology may decrease with increased age. Malikides *et al.* (2003) proposed that both TA and BAL samples ought to be collected in order to best investigate lower airway disease.

1.5.3.3 Sample Processing

Delays in processing of up to 8 hours will result in minimal cellular deterioration if samples are kept at room temperature and this may be extended to 24 hours if samples are refrigerated at 4°c (Pickles *et al.* 2002c). Samples for microbial culture should be processed more quickly to prevent bacterial overgrowth. Airway washes can be made into smears but are of low cellularity and are therefore easier to examine if they are centrifuged to concentrate cells prior to being re-suspended, or are cytocentrifuged. Although both of these procedures, especially cytocentrifugation, are used routinely, both procedures may

cause alterations in cellular morphology. Centrifugation may cause a decrease in mast cell percentage whilst cytocentrifugation may cause a decrease in lymphocyte percentage without affecting the total cell count (Lapointe *et al.* 1994). Cytocentrifuged samples generally contain higher macrophage and lower lymphocyte differential counts when compared to smear preparations, although in one study the differences were clinically non-significant and cytological examination of the cytospun samples was easier to perform (Pickles *et al.* 2002b). A variety of stains are commonly employed including Diff Quik, Wright-Giemsa and May-Grünwald stains. Specific stains may also be used, for example, non-specific esterase to differentiate immature macrophages from large lymphocytes, Perl's Prussian blue for haemosiderin and Leishmann's or Toluidine blue for the metachromatic granules of mast cells. Fast Romanowsky stains (for example Diff-Quik) are not suitable for identification of mast cells (Hughes *et al.* 2003).

1.5.3.4 Evaluation of Tracheal Aspirates and Bronchoalveolar Lavage Fluid (BALF)

Grossly, BALF samples may be assessed for turbidity, presence of flocculent debris, clot formation and colour. Normal samples are colourless and clear or only slightly turbid. Discolouration may indicate increased cellularity (leukocytes or erythrocytes) or contamination (Hodgson and Hodgson 2007). Turbidity is a feature of increased cellularity or increased protein concentration (Hodgson and Hodgson 2007). Increased mucus within tracheal aspirates is generally assumed to be a non-specific indicator of inflammation and subsequent cytological examination often reveals an increase in cellularity. Mucus may become inspissated and deeply basophilic staining or form airway casts (Curschmann's spirals) especially in chronic or suppurative disease. The significance of mucus in the absence of an increase in cellularity is uncertain (Hodgson and Hodgson 2007). The presence of atypical bronchial epithelial cells has been described as an indicator of airway pathology. The presence of squamous epithelia from the upper respiratory tract indicates contamination. Erythrocytes and haemosiderophages are an indicator of pulmonary haemorrhage, most commonly as a result of EIPH or iatrogenic damage during sampling. The presence of haemosiderophages indicates that haemorrhage did not occur at the time of sample collection.

Total nucleated cell counts (TNCC) may be increased in both infectious and non-infectious airway diseases. Total nucleated cell counts are influenced by dilution when fluid is infused and aspirated (in both TA and BAL). Efforts have been made to estimate the effects of dilution by correction according to the albumin or urea concentration (McGorum

et al. 1993f), however urea concentration may increase with pulmonary inflammation and confound the results. Retrieval of a set percentage of the infused saline has been recommended but is rarely possible (Hodgson and Hodgson 2007). Cellular clumping within mucus can result in a false decrease in TNCC; an effect that cannot be prevented by filtering of mucus as epithelial cells, macrophages and mast cells are also removed. Automated counters are inaccurate if mucus is present within the sample. Though published ranges are varied, both TA and BALF from normal horses contain <1 x 10^9cells/litre with few to no erythrocytes (Hodgson and Hodgson 2007). Intracellular organisms and pollen may be visible.

Differential leukocyte counts are widely accepted as the most valuable measures of inflammation in non-infectious airway disease yet there is variation in reported cut-off values for different conditions. Macrophages and lymphocytes make up the majority of cells whilst other cell types are usually present in low numbers.

Macrophages are the predominant cell type in both tracheal aspirates (TA) and BALF and increases are both difficult to detect and of uncertain significance (Hodgson and Hodgson 2007). Haemosiderophages may be present as a result of haemorrhage distal to the larynx and although presence in BALF correlates well with exercise-induced pulmonary haemorrhage (EIPH) they are also a normal finding in horses in training (McKane *et al.* 1993). Haemosiderophages may take months to be removed from the lower airways, making interpretation of their significance difficult and preventing any consensus on what constitutes a "normal" number (Hodgson and Hodgson 2007).

The variation in percentage of lymphocytes within TAs and particularly BALF is large and although increased percentages have been associated with exercise intolerance and chronic coughing, the significance of large numbers is uncertain (McGorum and Dixon 1994). It has been suggested that the lymphocytic inflammation that commonly occurs in young horses may bear some similarity to the acute form of hypersensitivity pneumonitis or farmer's lung resulting from chronic exposure to organic antigens (Viel and Hewson 2001).

Whilst RAO in horses is typified by airway neutrophilia, the condition of neutrophils may be the only means of differentiating infectious from purely inflammatory airway diseases; in RAO, IAD and EIPH neutrophils are typically mature with few degenerate or toxic changes. In contrast, the production of cytolysins by infectious agents often causes

neutrophil degeneration. Recurrent airway obstruction is characterised by BALF neutrophilia and whilst there is disagreement over cut off values between normal and abnormal BALF, it is more appropriate to consider >25% neutrophils indicative of RAO and <5% consistent with absence of pulmonary disease (Robinson 2001). Reported neutrophil percentages range from 0-28% in control horses (although in all but one study results were below 10%) and from 6-65% in horses with RAO (in all but one study results were over 29%) (Derksen *et al.* 1985b; Costa *et al.* 2000b; McGorum *et al.* 1993e; Tremblay *et al.* 1993). In horses with RAO it is not uncommon for neutrophil percentages to be as high as 98% (Lavoie 2007). However, neutrophil percentages in BALF do not necessarily correlate with clinical signs (Grunig *et al.* 1989). Some horses may show marked respiratory difficulty and have only mild (10-20%) airway neutrophilia (Lavoie 2007) whilst stabling of normal horses, both young and old, may result in airway neutrophilia (Tremblay *et al.* 1993; Holcombe *et al.* 2001). Outdoor housing and hay feeding has similarly been shown to result in airway inflammation (Robinson *et al.* 2006). Horses with RAO that are in remission may not have any cytological changes; although airway neutrophilia can be induced by as little as 5 hours of exposure to allergens. Diagnosis therefore necessitates interpretation of cytology findings in light of other clinical findings and clinical history.

Low numbers of eosinophils are a normal finding in equine airway secretions. Eosinophilia is typically considered a feature of parasitic and type I hypersensitivity diseases. Infection with *Dictyocaulus arnfeldi* (although rare) may cause a profound airway eosinophilia (MacKay and Urquhart 1979). In the absence of parasitic disease, eosinophilia (often accompanied by mastocytosis) may be assumed to be the result of a hypersensitivity response, however increases are often small and transient (<24h) and eosinophilia is rarely a feature of RAO (Lavoie 2007). Airway eosinophilia may be cytological evidence of eosinophilic pulmonary disease. Eosinophilia in the BALF of young racehorses has been associated with airway hyperreactivity (Hare and Viel 1998).

Mast cells are most often seen in BALF, however occasionally mast cells may be identified in tracheal aspirates (Mair 1987, Hughes *et al.* 2003). While large percentages of mast cells have been identified in normal horses (McGorum and Dixon 1994), increases have also correlated with increased hyperreactivity to histamine bronchoprovocation in horses with exercise intolerance (Hare *et al.* 1994; Hoffman *et al.* 1998). Some studies may have underestimated mast cell numbers by mistakenly relying on fast Romanowsky stains (McKane *et al.* 1993; Lapointe *et al.* 1994). The use of fast Romanowsky stains will result

in a significantly lower mast cell percentage compared to the use of a metachromatic stain (e.g. Leishmann's stain) (Hughes *et al.* 2003).

1.5.4 Diagnostic Imaging

1.5.4.1 Radiography

Thoracic radiography in adult horses is complicated by the size of the animal's chest and by the overlying structures of the forelimb. High exposures are often required to achieve sufficient x-ray beam penetration for acquisition of thoracic radiographs. Tissue contrast is increased by capturing the image at maximal inspiration. In order to achieve optimal timing with minimal movement, thoracic radiography is generally performed under standing sedation. Dorsoventral images cannot be obtained in the adult horse; radiography may be performed from both sides of the chest to allow the site of unilateral lesions to be determined. The trachea, lungs, main-stem bronchi, cardiac silhouette, aorta, pulmonary vasculature, caudal vena cava, mediastinum, diaphragm, ribs, vertebrae and sternum are all visible on thoracic radiographs. The major indication for thoracic radiographic examination is investigation of parenchymal disorders especially where disease of the deeper parenchyma is suspected. Radiographic investigation of the peripheral parenchyma may be valuable; however ultrasonographic examination is frequently more useful (Barakzai and McAllister 2007). Radiographic investigation is insensitive for the detection of thickening of the bronchi which is a characteristic pathology of both RAO and IAD (Christley and Rush 2007; Lavoie 2007). In cases of chronic RAO, a generalised increase in opacity may be evident with a bronchointerstitial pattern (Barakzai and McAllister 2007). Some authors have reported flattening of the diaphragm indicative of alveolar hyperinflation (McPherson *et al.* 1978; Seahorn *et al.* 1996). Air trapping within the distal airways may also result in less radiographic difference between inspiratory and expiratory radiographs than would be seen in normal horses (Farrow 2002). The major indication for thoracic radiography during investigation of RAO, SPARAO or IAD is the elimination of other possible diagnoses.

1.5.4.2 Ultrasonography

Normally aerated lung is highly echogenic and the interface of the pleura and aerated lung produces a reverberation artefact which prevents examination of deeper structures. In both RAO and IAD, the peripheral parenchyma remains either normally or over-inflated and has the same ultrasonographic appearance as normal lung. As with radiography, the major

indication of ultrasonographic examination in the investigation of RAO and IAD is the elimination of other conditions.

1.5.4.3 Nuclear Scintigraphy

Nuclear scintigraphy enables examination of regional ventilation (V), regional perfusion (Q), V:Q relationship, alveolar epithelial permeability, inflammatory cell distribution, mucociliary clearance and aerosol deposition within the respiratory tract (Votion *et al.* 1997a; Votion *et al.* 1997b; Votion *et al.* 1998; Votion *et al.* 1999a; Votion and Lekeux 1999; Votion and Lekeux 2003). Given alternate techniques are available for many of these analyses and the availability of nuclear scintigraphy is limited, it is a technique that is rarely utilised in the examination of the respiratory tract.

1.5.5 Lung Biopsy

In isolated cases of lower respiratory tract disease, diagnosis may require histopathologic examination of pulmonary tissue. Biopsy is generally performed for investigation of pulmonary infiltrative disease and is rarely required for diagnosis of lower airway inflammation. The least invasive means of harvesting tissue is collection of pinch biopsies of bronchial tissue under endoscopic guidance (Lugo and Peroni 2007), however this technique has not been adequately validated by comparison with established diagnostic methods. In one study of horses with RAO, bronchial mucosal biopsies were less likely to detect airway inflammation than percutaneous biopsies (Wilson *et al.* 1993). Percutaneous needle lung biopsy (Raphel and Gunson 1981) has become the standard method for collection of lung tissue as it is reasonably safe, easy to perform, inexpensive and is performed under standing sedation. Samples obtained may be small and may not be of diagnostic value (Lugo and Peroni 2007). However in one study, diagnosis was established in 54 of 66 horses with diffuse pulmonary disease via percutaneous biopsy (Pusterla *et al.* 2007). Complications of the procedure may include epistaxis, pneumothorax, pulmonary haemorrhage, tachypnoea and respiratory distress (Savage *et al.* 1998) but the rate of complications may be reduced by the use of spring-loaded automated biopsy devices (Venner *et al.* 2006). In a study of 66 horses with respiratory tract disease, complications were observed in only 6 cases using an automated device (Pusterla *et al.* 2007). Larger samples of lung tissue may be obtained via thorascopically guided wedge resection (Lugo and Peroni 2007). Whilst this technique is reported to be safe (Lugo and Peroni 2007) and has the advantage that the biopsy site may be visualised in order to check for haemorrhage,

the procedure is expensive and there are associated risks of collapsing and re-inflating the lung during thoracoscopy (Vachon and Fischer 1998).

1.5.6 Analysis of Markers in Blood

The results of haematological and serum biochemical examinations in horses with RAO or SPARAO are generally unremarkable (Couetil and Hinchcliff 2004; Lavoie 2007) and the main indication for performance of these analyses is to exclude other possible diagnoses. Results of haematological and blood biochemical analyses are normal by definition in cases of IAD. Small increases in fibrinogen may be observed in the acute stages of RAO (Couetil and Hinchcliff 2004; Borges *et al.* 2007). Horses with acute exacerbations of airway inflammation and horses with inflamed airways that are undergoing exercise may experience hypoventilation and ventilation perfusion mis-matching, resulting in hypoxia and less commonly hypercapnia that may be detected by collection of arterial blood samples (Dixon *et al.* 1995c; Couetil and Denicola 1999).

1.5.7 Allergy Testing

Some studies have identified differences in response to intradermal injection of histamine and fungal antigens between normal horses and horses with RAO (McPherson *et al.* 1979a; Wong *et al.* 2005). However, other studies have failed to demonstrate any difference following injection of allergens into the dermis or directly into the bronchi (McGorum and Dixon 1993; McGorum *et al.* 1993a; Lorch *et al.* 2001; Tahon *et al.* 2009) and these methods are not used in the diagnosis of allergic respiratory disease. Evaluation of IgE concentration in serum has also proved unhelpful in the diagnosis of RAO (Tahon *et al.* 2009).

1.5.8 Pulmonary Function Tests

The diagnostic tests outlined in sections 1.5.1 – 1.5.6 allow the clinician to establish whether lower airway inflammation is present and may give an indication of the severity of disease. However, in order to establish the functional consequences of disease, lung function testing is required. Pulmonary function testing (PFT) enables measurement of lung volume, mechanical function and the efficiency of gaseous exchange (Marlin and Deaton 2007). Specialist equipment and expertise is required for PFT and these procedures are mostly used in research settings. Standard PFT techniques such as measurement of

oesophageal pressure and flow rates may not detect differences between normal horses, horses with RAO that are in remission or horses with IAD. More sensitive techniques such as forced oscillation, forced expiration, volumetric capnography and plethysmography can be used to detect lower airway dysfunction in horses with IAD or sub-clinical RAO (Derksen *et al.* 1985a; Fairbairn *et al.* 1993a; Hoffman and Mazan 1999; Votion *et al.* 1999b; Couetil *et al.* 2001; van Erck *et al.* 2003; Andrew *et al.* 2007; Nolen-Walston *et al.* 2009).

1.5.9 Detection of Markers of Inflammation and Oxidative Stress

Pro-inflammatory oxidants are generated by metabolic enzymes, inflammatory cells and mitochondrial electron leakage. Endogenous and exogenous antioxidants counterbalance oxidative processes to maintain homeostasis. If homeostasis is not maintained, a state of oxidative stress may ensue in which cell damage may occur. Reactive oxygen species (ROS) or oxidants are oxygen-containing molecules that are more reactive than the oxygen molecule itself (Noguchi and Niki 1999). Reactive oxygen species may contain one or more unpaired electrons and are known as free radicals; for example, nitric oxide (NO^{-}), superoxide ($O_2^{\cdot -}$) and the hydroxyl radical (HO^{-}). Species that do not contain un-paired electrons are known as non-radical oxidants and include peroxynitrite ($ONOO^{-}$), hydrogen peroxide H_2O_2 and hypochlorous acid (HOCl). Antioxidants may be components of systems that prevent ROS generation, inactivate ROS or limit the deleterious effects of oxidative injury (Cheeseman and Slater 1993).

Measurement of free radicals is rarely possible due to the complexity of the laboratory processes and equipment required. Measurement of markers of oxidative damage and measurement of pro-oxidant elements and anti-oxidants is more commonly performed (Kirschvink *et al.* 2008). However, measurement remains largely restricted to research institutions. Ascorbic acid is the principal non-enzymatic anti-oxidant in pulmonary epithelial lining fluid (PELF) accompanied by glutathione (GSH) and uric acid (Art *et al.* 1999; Deaton *et al.* 2002; Deaton *et al.* 2005a). Nucleated cells present within PELF also contain superoxide dismutase, glutathione peroxidise and catalase (Deaton 2006).

Increased concentrations of reduced glutathione (GSH), oxidised glutathione (GSSG), 8-isoprostane, myeloperoxidase (MPO) and reduced levels of ascorbic acid have been identified in pulmonary epithelial lining fluid (PELF) in horses with RAO; as have increased levels of GSH and GSSG in erythrocytes RAO (Art *et al.* 1999; Kirschvink *et al.* 2002b; Deaton *et al.* 2004a; Art *et al.* 2006). Nucleated cells in PELF collected from

horses with RAO have higher activities of the enzymatic anti-oxidants catalase and glutathione peroxidise than control horses (Kramaric *et al.* 2005). Concentrations of oxidative markers and antioxidants have been shown to correlate with neutrophil percentages in BALF, indicating that inflammatory burst may be the major contributor to ROS production (Kirschvink *et al.* 2002b; Deaton *et al.* 2004a; Deaton *et al.* 2004b; Art *et al.* 2006). Initial induction of neutrophilic airway inflammation is not generally associated with detectable pulmonary oxidative stress and a sustained inflammatory response is considered necessary to deplete antioxidant capacity (Deaton 2006). Oxidant markers also correlated with measurements of lung function in RAO-affected horses (Kirschvink *et al.* 2002b), however the mechanisms by which oxidative stress may induce the pathophysiological mechanisms of RAO are unknown (Deaton 2006). Pulmonary oxidative stress is also a factor in human respiratory diseases including asthma and COPD (Rahman *et al.* 2000; Rahman 2005).

Horses with RAO have been supplemented with antioxidants including vitamin E, ascorbic acid, selenium and natural flavenoids in an effort to reduce oxidative stress in these animals (Deaton *et al.* 2002; Kirschvink *et al.* 2002a). Deaton *et al.* (2002) demonstrated that trace element supplementation resulted in increased levels of these antioxidants in plasma and that PELF ascorbic acid concentrations were increased. However, in another study, supplementation of anti-oxidants failed to result in any improvement in lung function variables (Kirschvink *et al.* 2002a).

1.5.10 Exhaled Breath and Breath Condensate Analysis

Collection of exhaled breath is a method described for diagnosis and monitoring of lower airway disease. The procedure is non-invasive and can be performed frequently without causing distress to the patient. Exhaled breath condensate (EBC) has been studied widely in the investigation of human respiratory diseases, most notably asthma (Jobsis *et al.* 1997; Ho *et al.* 1999; Hanazawa *et al.* 2000; Niimi *et al.* 2004; Brunetti *et al.* 2006). There are many constituents of exhaled breath condensate that may be influenced by lower airway inflammation including hydrogen peroxide (H_2O_2), nitric oxide, adenosine, arachidonic acid metabolites, cytokines, 8-isoprostane and ammonia (Horvarth *et al.* 2005). Hydrogen peroxide is one of the most widely used analytes in man (Horvath *et al.* 2005) and is primarily produced by activated inflammatory cells in the airways as a result of oxidative stress (Babior 2000).

Analysis of H_2O_2 concentration is complex as it is photo- and thermo-sensitive, reacts readily with a wide variety of chemicals and degrades rapidly (Duz *et al.* 2009). Despite these biological characteristics, concentration of H_2O_2 in EBC has been shown to correlate with presence and severity of asthma (Antczak *et al.* 2000; Loukides *et al.* 2002) and pH has been demonstrated to be a reliable indicator of inflammatory respiratory disease in man (Kostikas *et al.* 2002; Vaughan *et al.* 2003b; Borrill *et al.* 2005).

There are few studies of the collection and analysis of EBC from horses. In one study, H_2O_2 concentration in EBC was correlated with ascorbic acid concentration in PELF (Deaton *et al.* 2004b). However, further studies which compared horses with airway inflammation to normal controls failed to find significant differences in exhaled H_2O_2 concentrations (Deaton *et al.* 2005a; Wyse *et al.* 2005). Prolonged exposure to airway inflammation may be required to deplete antioxidant capacity (Deaton 2006).

Duz *et al.* (2009) reported a method of EBC collection that was well tolerated by horses. In that study, no significant differences in EBC hydrogen peroxide and pH between horses with mild lower airway inflammation and normal horses were found (Duz *et al.* 2009), however a trend for a decrease in EBC pH was detected. Using current methods, analysis of EBC is not reliable in the detection of lower airway inflammation and further development and research is required (Wyse *et al.* 2004; Duz *et al*. 2009).

1.6 Study Aims

The aim of the study was to investigate the expression of HO-1 and iNOS in the equine respiratory tract. The primary objective was to investigate whether HO-1 and iNOS were expressed within leukocytes from equine BALF and to quantify expression in different cell types.

If it proved possible to characterise and quantify expression then additional aims were to investigate:

1. Associations between HO-1 and iNOS expression and clinical signs of respiratory disease
2. Possible relationships between induction of HO-1 and iNOS
3. Associations between HO-1 and iNOS expression and markers of oxidative stress in EBC

1.7 Study Hypotheses

It was hypothesised that:

1. HO-1 and iNOS would be expressed in leucocytes from equine BALF and would be expressed most strongly in neutropils and macrophages.
2. Expression of HO-1 and iNOS would correlate positively with recognised markers of lower airway inflammation.
3. Expression of HO-1 and expression of iNOS would be correlated positively with one another in leucocytes from equine BALF.
4. Expression of HO-1 and iNOS in leucocytes from BALF would be higher in horses with higher concentrations of H_2O_2 and lower pH in exhaled breath condensate.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals

All investigations were performed with the consent of The Ethics and Welfare Committee, University of Glasgow. All horses over 12 months of age presented to the Weipers' Centre for Equine Welfare at The University of Glasgow or The Liphook Equine Hospital, Hampshire for the investigation of suspected lower respiratory tract disease between September 2004 and September 2007 were included. All horses were examined by the author. Horses of less than 12 months of age were excluded from the study.

2.2 Clinical Examination

Clinical examination of each horse was performed and results were recorded on a standard examination form developed prior to the start of sample collection (Appendix 1).

The age, breed, and gender of each horse included in the study were recorded. Prior to specific examination of the respiratory tract the animal's condition score was graded on a 1-5 scale (Carroll and Huntington 1988). Mucous membrane colour was recorded as grey, pale pink, pink, dark pink, red or toxic and capillary refill time was assessed subjectively and recorded to the nearest second. Heart rates were recorded during auscultation of the heart. Rectal temperature was recorded in °C.

The pattern and nature of breathing was assessed from a distance and scored according to a formula reported previously (Seahorn *et al.* 1997). The degree of medial and lateral nostril flare and the degree of abdominal lift was used to determine a single score calculated for each horse (Seahorn *et al.* 1997). The nares were examined for evidence of nasal

discharge. The quantity of any discharge was recorded as nil, scant, moderate or profuse. The appearance of any discharge was noted as serous, mucopurulent, purulent, brown/beige or blood-tinged. The owners of horses included in the study were questioned as to whether the horse had a history of nasal discharge or coughing. According to owner responses, the frequency of coughing was recorded as occasional, frequent single, frequent paroxysmal or at exercise only. Further, coughing was classified as productive or non-productive and dry or moist. Historical data was recorded but was not included in statistical analysis.

Both lung fields and the trachea were auscultated and the presence of any wheezes (indicative of bronchoconstriction) and/or crackles (indicative of fluid in the distal airways) were recorded. Auscultation was repeated using a re-breathing bag in order to increase the sensitivity of detection of abnormal breath sounds and findings were recorded.

Endoscopy was performed in all horses. Horses examined at The University of Glasgow were examined using a 1.4m videoendoscope (Stortz, Slough, UK). Horses examined at The Liphook Equine Hospital were examined using a 1.5m videoendoscope (EV Veterinary Products, Shropshire, UK). If the trachea was judged to be erythematous, this was noted, however data were not subjected to statistical analysis as more reliable and objective means of assessing lower airway inflammation were used (BALF cytology). The degree of tracheal mucus accumulation was graded according to a scale reported previously (Gerber *et al.* 2004b). The presence of oedema around the carina was recorded; however, during the study period Koch *et al.* (2007) demonstrated this observation was not useful for assessment of lower airway inflammation and data were not included in statistical analysis.

Tracheal aspirates were performed in the majority of horses included in the study using a standardised protocol. In brief, twenty millilitres of phosphate buffered saline (PBS) was instilled into the trachea trans-endoscopically and then aspirated from the tracheal sump via the same catheter. Detomidine (0.01mg/kg, intravenously) and butorphanol (0.02 mg/kg, intravenously) were used to facilitate endoscopy and subsequent bronchoalveolar lavage as required.

2.3 Bronchoalveolar Lavage

Bronchoalveolar lavage was performed in all horses using a commercially available cuffed BAL tube from Cook Veterinary Products (Bloomington, Indiana, US) or Bivona (Gary, Indiana, US). The diameters of both tubes were identical. In all horses, BAL was performed using the same protocol. The BAL tube was lubricated with a small quantity of sterile, water-soluble lubricant and passed via the ventral meatus into the trachea until it wedged in a bronchus. The cuff of the tube was then inflated with 5 ml air and 300ml of PBS in 6 x 50ml syringes was instilled as rapidly as possible into the distal airways. The saline was then withdrawn via the tube and the samples in each syringe pooled to give a single sample. Volumes of fluid collected were recorded.

2.4 Fluid Processing

The pooled sample of BALF was mixed by repeated inversion and 2ml aliquots were removed using a sterile syringe. Aliquots from all samples were snap frozen in liquid nitrogen within 5 minutes of collection and stored either in liquid nitrogen or in a freezer at -80°C for subsequent processing. For each BALF sample collected at the University of Glasgow, one aliquot was frozen and one aliquot was refrigerated and processed within 4 hours of collection. In the majority of BALF samples, processing was performed within one hour, although previous studies have demonstrated that results of cytological examination are not affected if samples are processed within 4 hours (Pickles *et al.* 2002c). Frozen BALF was thawed at laboratory temperature (approximately 22^{o}C) prior to slides being prepared as they were for fresh BALF. Total cell counts were performed using an automated analyser. Samples were then cytospun at 1500 rpm for 5 minutes. Six slides were produced for each fresh and frozen sample to provide slides for cytological and immunocytochemical examination from identical sample aliquots. Slides for cytological examination were stained using Leishmann's and Giemsa stains and leukocyte differential counts were calculated following examination of 200 cells. The remaining slides were air dried and stored for subsequent immunostaining.

2.5 Immunocytochemistry

Cytospins were prepared prior to immunocytochemical staining taking place and stored at 4ºC until staining with either iNOS (Biomol International, Exeter, UK) or H0-1 (Biomol

International, Exeter, UK) antibody. Both antibodies used were rabbit polyclonal and staining was performed using Dako EnVision kit K4011 (Dako, Ely, UK) as per manufacturer's instructions with all incubations at room temperature.

The cytospins were placed initially in 10mM Tris buffer saline pH 7.5 with Tween (TbT; Dako, Ely, UK) for 5 minutes prior to application of EnVision Peroxidase block (Dako, Ely, UK) in order to quench endogenous peroxidases for 5 minutes. The sections were washed twice for 5 minutes with TbT before application of designated antibody at a previously optimised dilution for 1 hour. Both optimal antibody dilutions were 1/50 with the antibodies diluted using Dako Real antibody diluents (Dako, Ely, UK). The sections were washed twice for 5 minutes with TbT before application of the labeled polymer-HRP anti-rabbit signal amplification step for 30 minutes. The sections were then washed twice for 5 minutes and 3,3' diaminobenzidine chromogen solution applied for 10 minutes in order to allow visualisation of the epitope-antibody interaction. This reaction was terminated by placing the sections in de-ionised water. The sections were then counterstained by transferring into Gill's Haematoxylin (4 minutes), differentiated in 1% acid alcohol (3 dips) and then the nuclei blued in Scotts tap water substitute (1 minute). After washing in running tap water the sections were taken through graded alcohol (70%, 100% and 100% ethanol) then xylene (x3) before application of a coverslip and permanently mounting using a distyrene, plasticizer, and xylene (DPX) mountant for microscopy.

2.6 Examination of Immunostained Slides

Slides were examined under a light microscope at 40-100 x magnification. Representative regions of each slide were selected and images captured using a digital camera connected to the microscope. One hundred cells were examined for each slide. If insufficient cells were present in a single image capture then further captures were taken from the same slide and if necessary, from further slides made from the same aliquot until in excess of 100 cells were present or all cells on the slides had been examined. All cells on a capture were counted to ensure that the count was objective and representative. Counts were facilitated by the use of automated counting software used in association with Adobe Photoshop (CS2 Version 9; Adobe Systems Incorporated, USA).

Cells in fresh samples were classified as neutrophils, macrophages or lymphocytes on the basis of morphology. Differentiation of immunostained eosinophils and mast cells was

found to be impossible. It was intended that cell morphology would be determined and staining graded for frozen samples in the same manner. However, cell morphology was disrupted markedly by the freezing and thawing of cells and distinction between cell types was not possible. Assessment of frozen samples was performed by considering cells collectively without differentiation into specific cell types.

The results of pilot studies indicated that in virtually all cells uptake was diffuse throughout the cytoplasm of the cell and distributed equally throughout the cell. The degree of staining for each cell was graded on 0-4 scale:

- Grade 0: No stain uptake
- Grade 1: Stain visible but barely discernable
- Grade 2: Pale brown staining
- Grade 3: Dark brown staining

On the rare occasion that stain uptake was not equal throughout the cytoplasm an average of the degree of staining across the cell was taken.

2.7 Collection of Exhaled Breath Condensate

Exhaled breath condensate (EBC) was collected according to a method described previously (Duz *et al.* 2009). Collection of EBC was performed before horses were sedated for the BAL procedure. A face mask was connected via tubing to a polypropylene condensation chamber immersed in ethanol/liquid nitrogen slurry to maintain a temperature below -80°C. The face mask was constructed from a muzzle modified with fibreglass and incorporated two one-way non-rebreathing valves. A rubber diaphragm around the mask created an airtight seal with the horse's head. The non re-breathing valves were connected by a rubber flexible tube (Cranlea, UK) to the condensation chamber. Exhaled breath condensed on the internal surface of the polypropylene container. A one-way valve on the outlet of the condensation chamber prevented contamination of the sample with environmental air. Breath condensate was collected over a 10 minute time period. Collected samples of EBC were maintained on ice during transit to the laboratory.

2.8 Analysis of Exhaled Breath Condensate

Exhaled breath condensate was thawed and analysed immediately. Measurement of H_2O_2 concentration was performed using a spectrophotometric assay based on the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of horseradish peroxidise (HRP; Sigma-Aldrich, UK) modifying a published method (Gallati and Pracht 1985). In summary, 15 µl of HRP (75IU/ml), 140 µl of TMB in 0.42M citrate buffer (pH 3.8) and 140 µl of EBC were mixed at room temperature. The reaction was stopped after 10 minutes by addition of 15µl of 9M sulphuric acid. The product of the reaction was analysed immediately with an automated microplate reader (FLUOstar OPTIMA, BMG labtech, Aylesbury, UK) using a wavelength for absorbance of 450 nm. Exhaled breath condensate H_2O_2 concentration was calculated from a standard curve of known concentrations of H_2O_2 (from 0.1 to 10 µM) prepared fresh for each assay. The lower detection limit of the assay was 0.1 µM. Linearity of the standard curve was acceptable (r^2 > 99%) between 0 and 10 µM. The measurement was repeated 4 times for each sample and the final H_2O_2 concentration was calculated as the mean of the 4 measurements.

Measurement of pH of EBC samples was performed using a pH meter (pH21, Hanna Instruments Ltd) connected to a glass-body pH electrode with an accuracy of ± 0.02 pH (HI-2031B, Hanna Instruments Ltd).

2.9 Case definitions

In accordance with the guidelines published in the consensus statements pertaining to RAO and IAD, the results of cytological analysis of BALF were considered central to the diagnosis of lower airway inflammation (Couëtil *et al.* 2007; Robinson 2001). Pulmonary function testing was not available. Cut-offs were based upon the findings of studies of BALF from normal horses (Table 2.9) and values published previously (Mair *et al.* 1987; Grunig *et al.* 1989; Vrins *et al.* 1991; Naylor *et al.* 1992; Lapointe *et al.* 1993; McGorum *et al.* 1993e; Jean 1996; Robinson 2001; Couëtil *et al.* 2007). Neutrophilia was used to quantify airway inflammation; horses with greater than 5% neutrophilia in BALF were considered to have inflammation of the distal airways. Horses with less than 5% neutrophils in BALF were considered to be free of lower airway inflammation. Relative

increases in eosinophil and mast cell counts were noted but were not used to define the presence of inflammation as they are an inconsistent finding in horses with lower airway inflammation (Derksen *et al.* 1985b; Derksen *et al.* 1989; Vrins *et al.* 1991; Winder *et al.* 1991). Following demonstration of the presence of airway inflammation, clinical and historical data were used to determine whether horses were likely to be suffering from IAD or RAO. For example, horses that demonstated clinical signs at rest or in response to environmental allergens were considered to be suffering from RAO. Horses that were investigated for performance, did not have marked neutrophilia in BALF and failed to exhibit signs at rest were considered more likely to be suffering from IAD.

Table 2.9. Published values for differential cell counts in equine BALF (mean values).

	McGorum *et al.* 1993	Mair *et al.* 1987	Naylor *et al.* 1992	Lapointe *et al.* 1994	Vrins *et al.* 1991
Neutrophil (%)	1.4	6.2	4.4	2.4	3.6
Macrophage (%)	58.5	70.3	48.2	25.6	65.1
Lymphocyte (%)	31.8	7.6	38.8	71.4	28.9
Eosinophil (%)	0	1.0	1.3	0.0	0.1
Mast Cell (%)	7.4	0.6	7.3	0.5	1.4

2.10 Data Analysis

Data were collated and processed using Microsoft Excel (Microsoft Office 2007, Microsoft Corporation, Richmond, USA). Statistical analysis was performed using Minitab (Minitab Ltd, Pennsylvania, USA). Grouped data were compared using Pearson's correlation when data were continuous. Categorical data sets were compared using the Mann-Whitney test. Significance was assumed where $p<0.05$. Results were also plotted graphically to aid interpretation.

Intra-observer agreement was investigated by calculation and graphical representation of the means and standard deviations for repeated analysis.

CHAPTER THREE

CHARACTERISATION OF EXPRESSION OF HO-1 AND iNOS WITHIN INFLAMMATORY CELLS OF FRESH AND FROZEN EQUINE BALF

3.1 Introduction

Haem oxygenase-1 is a heat shock protein responsible for the degradation of haem into bilirubin, carbon monoxide and iron (Tenhunen *et al.* 1969). The significance of HO-1 in respiratory disease is not known, however there is extensive evidence that the products of HO are protective against tissue damage which may be induced by inflammation and oxidative stress (Vogt *et al.* 1995; Otterbein *et al.* 1999b; Christou *et al.* 2000; Hashiba *et al.* 2001; Stocker 2004). Carbon monoxide in particular has been pursued as a possible therapeutic intervention for human lung injury (Otterbein *et al.* 2000; Otterbein 2002). Haem oxygenase-1 has been identified in equine lung tissue and its induction may have implications for respiratory physiology and pathology (Wyse *et al.* 2006). Further characterisation of the expression of HO-1 would be a useful step to help elucidate the importance of this enzyme in equine respiratory health and disease.

Nitric oxide, produced by nitric oxide synthase (NOS) has been implicated as a mediator of airway inflammation in human asthma and is produced in large quantities from bronchial epithelial and inflammatory cells of affected patients (Barnes and Liew 1995). Potentially deleterious effects of NO and RNS include vasodilation and plasma extravasation, increased airway secretions, impaired ciliary motility, increased Th2-mediated eosinophilic inflammation, necrosis and apoptosis (Barnes and Liew 1995). In horses, expression of inducible iNOS was found to be greater in the bronchial epithelium of animals with SPARAO than in unaffected animals (Costa *et al.* 2001). Nitric oxide produced by the activity of iNOS may therefore be an important mediator in equine lower airway

inflammation and further characterisation of the expression of iNOS would be a step forward in understanding its potential roles and significance.

Following collection of fluid samples from the equine respiratory tract, laboratory facilities may not always be available to permit immediate immunocytochemical examination. The equipment and skills required for cytospin preparations and immunostaining are generally restricted to specialist laboratories. The effects of delays in the analysis of bronchoalveolar lavage fluid have been investigated (Pickles *et al.* 2002a, 2002b, 2002c). In addition, immunostaining commonly results in variation in the appearance of cells between batches and these variations are exaggerated when equipment and personnel are not standardised (C.Nixon, personal communication). It would be advantageous if samples could be frozen and stored for subsequent processing for cytological and immunocytochemical examinations. This had particular relevance to our study as a cytospin was not always available to allow immediate preparation of slides from fresh BALF.

3.2 Aims

To determine whether there is good agreement in immunocytochemical examination findings of both HO-1 and iNOS expression between leukocytes in fresh and frozen aliquots of BALF samples obtained from horses. This would allow the results of immunostaining from frozen samples to be utilised in later investigations. It was hypothesised that there would be no significant difference between the results of immunostaining fresh and frozen BALF.

3.3 Methods

Samples of BALF were collected and processed as described in Chapter 2.3. In brief, following sedation with detomidine and butorphanol, BALF was collected using a commercially available BAL tube. Phosphate buffered saline in 6 x 50ml aliquots was instilled as rapidly as possible into the distal airways. The saline was then withdrawn via the tube and the samples in each syringe pooled to give a single sample. Aliquots of 2ml were obtained from the pooled sample and slides were prepared from one single aliquot within 4 hours of collection. A further aliquot was snap frozen in liquid nitrogen within 5 minutes of collection and then transferred to a freezer within which the temperature was maintained at -80°c. Frozen samples were subsequently thawed at room temperature (around 22°C) and then processed in the same manner as fresh samples. The slides were

stained for iNOS and HO-1 using rabbit polyclonal antibodies utilizing a Dako EnVision kit K4011 (Dako, Ely, UK) as per manufacturer's instructions and all incubations were performed at room temperature as outlined in Chapter 2.

Stained BALF from 12 horses was examined as described in Chapter 2. In short, during examination of each slide, the degree of staining was determined for each cell. Representative regions of each slide were selected and images captured using a digital camera connected to the microscope (Figure 3.3.1). One hundred cells were examined for each slide. If insufficient cells were present in a single image capture then further captures were taken from the same slide and if necessary, from further slides made from the same aliquot until in excess of 100 cells were present or all cells on the slides had been examined. All cells on a capture were counted to ensure that the count was objective and representative. For samples that were prepared before freezing, cells were classified as neutrophils, macrophages or lymphocytes. Pilot data had indicated that staining of mast cells and eosinophils was inconsistent and that these cell types were difficult to identify with confidence following immunostaining. They were, therefore, not included in the counts.

Examination of slides made from samples that had been frozen revealed changes in cell morphology had occurred that prevented reliable identification of cell types (Figure 3.3.1). Comparison of the images in figure 3.3.1 illustrates the differences between fresh and frozen samples. For frozen samples, nucleated cells were not differentiated by cell type and were considered collectively. The quality of iNOS staining for four samples was very poor and these slides were excluded from analysis (Figure 3.3.2). In order to allow statistical comparisons to be performed the results of the counts for each cell type in fresh BALF were combined to give total numbers of cells staining each grade (i.e. the same as for the frozen samples). Percentage of cells positive for immunocytochemical staining for fresh and frozen aliquots of BALF samples were compared using Pearson's correlation.

Figure 3.3.1 Haem oxygenase-1 staining in a cytospin prepared from fresh (left) and frozen (right) BALF collected from Horse 11.

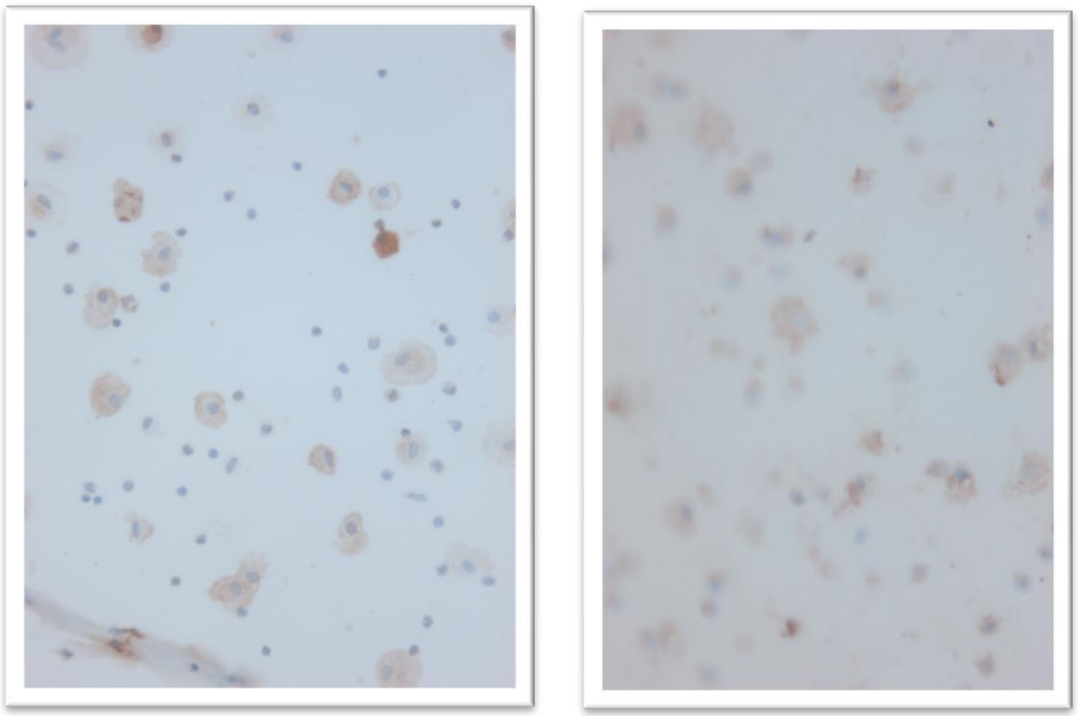
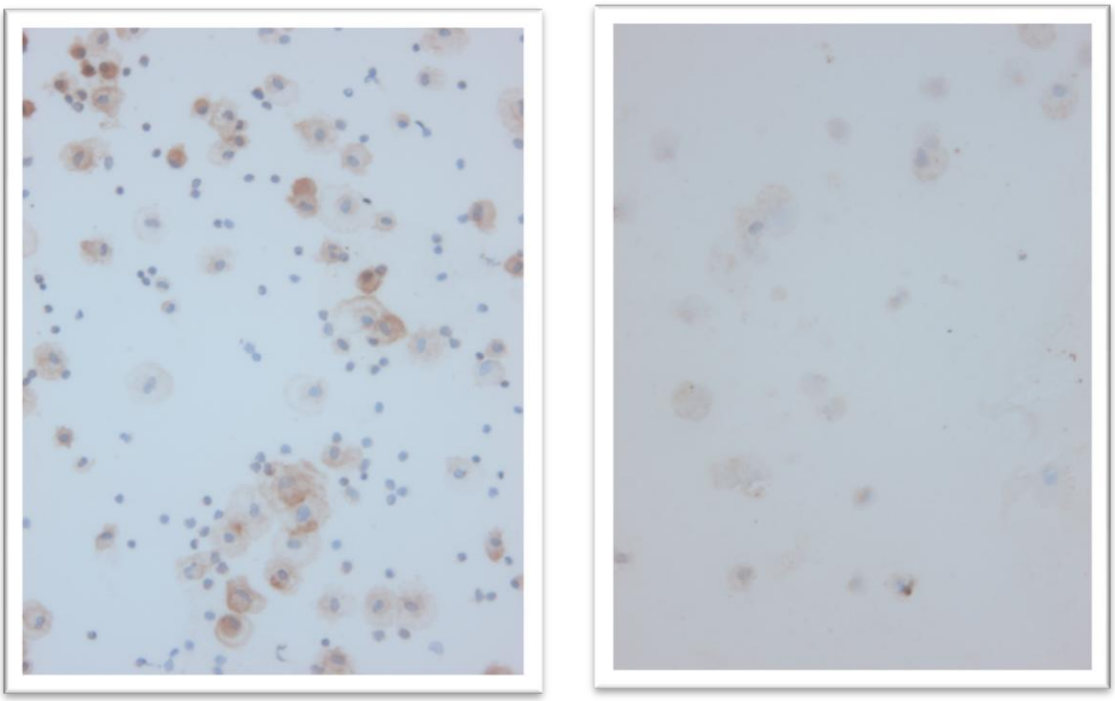


Figure 3.3.2. Inducible nitric oxide synthase staining in a cytospin prepared from fresh (left) and frozen (right) BALF collected from horse 11.



3.4 Results

The results of staining for fresh and frozen BALF are presented in Table 3.4.1 – 3.4.4 and the results of statistical analysis of the data are presented in Table 3.4.5. Only one statistically significant correlation was identified; cells staining grade 3 for iNOS. The results for fresh and frozen samples were displayed graphically in figures 3.4.1 and 3.4.2. The random distribution of the data points illustrates the lack of correlation between the results for fresh and frozen samples.

Table 3.4.1 Results of staining for HO-1 in frozen and thawed BALF from 12 horses.

Horse	Number of cells staining grades 0-3 (percentage of the total number of cells in brackets)							
	0	0(%)	1	1(%)	2	2(%)	3	3(%)
1	36	30	47	39	37	31	0	0
2	10	9	28	25	72	65	0	0
3	44	42	30	28	30	28	0	0
4	3	3	17	17	21	21	59	59
5	85	85	15	15	0	0	0	0
6	13	12	56	50	42	38	0	0
7	95	95	5	5	0	0	0	0
8	25	25	36	35	42	41	0	0
9	97	97	3	3	0	0	0	0
10	55	55	45	45	0	0	0	0
11	1	1	92	87	13	12	0	0
12	2	2	64	53	55	45	0	0

Table 3.4.2 Results of staining for HO-1 in fresh BALF from 12 horses.

Horse	Number of cells staining grades 0-3 (percentage of the total number of cells in brackets)							
	0	0 (%)	1	1 (%)	2	2 (%)	3	3 (%)
1	2	2	50	38	79	59	2	2
2	30	30	20	20	25	25	26	26
3	23	19	16	13	18	15	65	53
4	42	36	21	18	51	44	3	3
5	17	23	37	51	19	26	0	0
6	48	41	42	36	24	21	2	2
7	15	12	0	0	47	39	59	49
8	48	37	48	37	19	15	14	11
9	35	38	40	43	10	11	7	8
10	0	0	16	15	89	85	0	0
11	71	53	31	23	29	21	4	3
12	29	25	33	28	42	36	13	11

Table 3.4.3 Results of staining for iNOS in frozen and thawed BALF from 9 horses.

Horse	Number of cells staining grades 0-3 (percentage of the total number of cells in brackets)							
	0	0(%)	1	1(%)	2	2(%)	3	3(%)
2	28	27	44	43	30	29	0	0
3	28	28	34	34	19	19	20	20
4	17	15	40	34	59	51	0	0
5	100	100	0	0	0	0	0	0
6	63	52	20	17	38	31	0	0
8	43	35	56	45	25	20	0	0
9	50	50	50	50	0	0	0	0
10	50	50	50	50	0	0	0	0
12	78	61	50	39	0	0	0	0

Table 3.4.4 Results of staining for iNOS in fresh BALF from 9 horses.

Horse	Number of cells staining grades 0-3 (percentage of the total number of cells in brackets)							
	0	0 (%)	1	1 (%)	2	2 (%)	3	3 (%)
2	57	54	15	14	29	28	4	4
3	7	6	26	21	23	19	65	54
4	7	6	14	12	92	81	0	0
5	30	28	77	72	0	0	0	0
6	35	32	45	41	24	22	5	5
8	8	7	59	48	46	38	9	7
9	41	21	50	25	108	54	0	0
10	0	0	60	35	101	59	9	5
12	7	5	63	44	69	48	4	3

Table 3.4.5 Results of Pearson's Correlations comparing the results of staining (performed on percentage values) for fresh and frozen cells. Significant associations highlighted in bold.

	HO-1		iNOS	
	r value	p value	r value	p value
Cells staining 0	-0.370	0.237	0.113	0.772
Cells staining 1	0.004	0.991	-0.602	0.086
Cells staining 2	-0.240	0.452	0.250	0.516
Cells staining 3	-0.189	0.557	0.988	**0.000***

Figure 3.4.1 Scatter plot showing the results of staining fresh and frozen BALF cells from 12 horses.

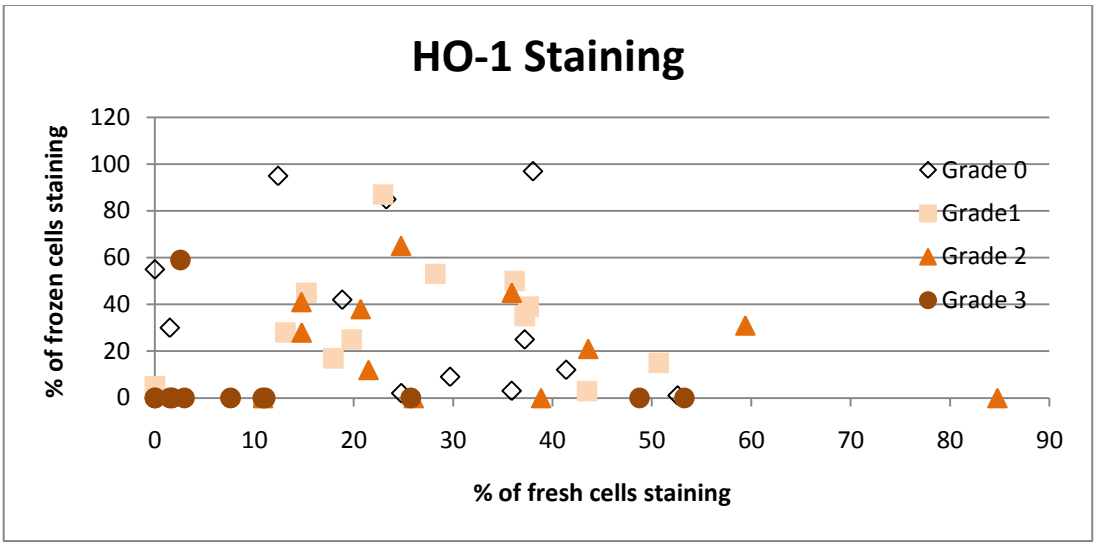
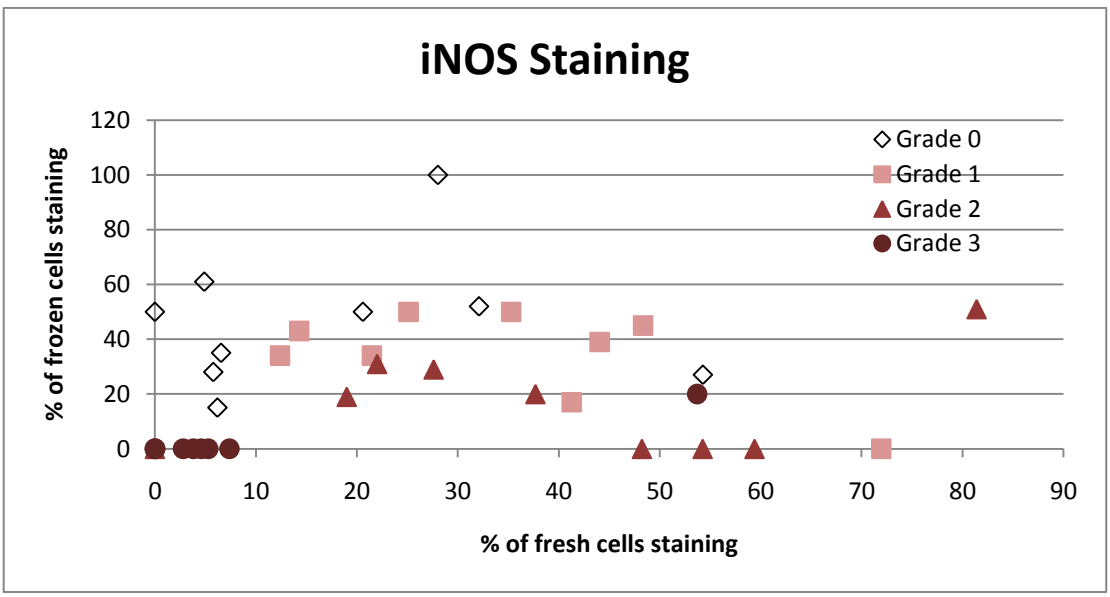


Figure 3.4.2 Scatter plot showing the results of staining fresh and frozen BALF cells from 12 horses.



3.5 Discussion

A significant correlation was determined in only 1 or the 6 tests of correlation performed; cells staining grade 3 for iNOS. For this type and degree of staining the correlation was not only significant but was linear with the r value being 0.988. However, this result was strongly influenced by the presence of a single strong positive result (case 3) and a large number of negative results. Overall, correlation was poor and as the preservation of the fresh samples was visibly superior it was assumed that error was introduced by freezing and thawing. When fresh and frozen cells were compared the frozen cells were smaller and irregular in appearance. Cells were commonly aggregated and distinction of individual cells proved difficult. The subjective assessment of staining in individual frozen cells was therefore less precise. Background staining was also greater in the slides of frozen BALF. A degree of background staining is expected with the immunocytochemical methods utilised (C.Nixon, personal communication) as was observed in the slides of fresh BALF. However, with the comparatively large amount of background staining of the frozen samples and the shrunken appearance of the frozen cells we suspected that constituents of the cytoplasm were contributing to the background staining. The freezing and thawing processes are suspected to have damaged the phospholipid cell membrane.

We rejected the hypothesis that there would be no significant difference between the results of immunostaining fresh and frozen BALF. Frozen BALF would appear to be unsuitable for immunocytochemistry and the results from cases in which only frozen data was available were omitted from further analysis within this collection of studies. These results are presented in Appendix 3. For immunocytochemical staining of cells in BALF, processing of samples should be performed shortly after collection of BALF without freezing.

Alternatively, cryopreservation techniques could, and in this study should, have been performed. Conventional cryoprotectants are glycols, such as ethylene glycol, propylene glycol and glycerol. In molecular biology, glycerol is the most commonly used glycol; dimethyl sulfoxide (DMSO) is even more widely utilised and is also regarded as a conventional cryoprotectant.One method is the addition of DMSO to a final concentration of 10% followed by slow freezing (Pegg 2007). Cells may then be preserved frozen at -80°C or stored in liquid nitrogen. A controlled rate cooling process is preferable to allow biological samples to equilibrate in the cryoprotectant gradually and particularly to permit sufficient water to leave the cell during progressive freezing of the extracellular fluid. Optimal rate of freezing varies between cells of differing size and water permeability: a

typical cooling rate of around 1°C per minute is appropriate for many mammalian cells after treatment with cryoprotectants such as dimethyl sulphoxide. Foetal bovine serum may also be added to 10% DMSO to enhance the survival of some cell lines. In our study cells might have been preserved successfully if BALF had been centrifuged and the cell pellet resuspened in a mixture of DMSO and foetal bovine serum. However, FBS carries the risk of infection by abnormal prion proteins and viruses serum-free cryopreservative solutions have been developed recently. A sericin cryopreservative solution consisting of phosphate-buffered saline, 1% sericin, 0.5% maltose, 0.3% proline, 0.3% glutamine and 10% DMSO has recently been shown to be effective (Toyosawa *et al*. 2009).

In a previous study cytospin slides were prepared and frozen for subsequent thawing and immunostaining (Horvath *et al.* 1998). The limiting factor in our investigations was the availability of a cytospin and hence this method of sample storage offered no advantage. The effects of a delay between slide preparation and immunostaining were not investigated in this study. It is unknown whether it is possible to store air-dried slides prior to immunostaining.

CHAPTER FOUR

INTRA –OBSERVER AGREEMENT OF THE ASSESSMENT OF IMMUNOSTAINING INFLAMMATORY CELLS FROM EQUINE BALF

4.1 Introduction

Immunostaining has been used previously to identify the presence of HO-1 in equine tissues fixed in formalin and embedded in paraffin (Wyse *et al.* 2006). Expression was identified, on the basis of cell morphology, within alveolar type II epithelial cells, neutrophils and macrophages. Immunostaining has also been used to identify the presence of iNOS within paraffin embedded sections of lung and other equine tissues (Mirza *et al.* 1999; Costa *et al.* 2001; Mirza *et al.* 2005). Immunostaining of cells contained within BALF for iNOS and HO-1 has not, to the author's knowledge, been performed previously. In all previously reported studies rabbit antibodies directed against the target protein (either HO-1 or iNOS), have been used to induce a colour change and in doing so, label cells containing the target protein. Identification of immunostaining is dependent upon the operator noting a subjective colour change. It is important that the subjective methods used to quantify the degree of staining within each sample give a true and repeatable indication of expression. If the methods used to quantify immunostaining proved to be repeatable then they would be used to further characterize HO-1 and iNOS expression within equine BALF.

4.2 Aims

To determine whether the methods used to assess the degree of immunostaining in leukocytes collected from equine BALF were repeatable. We hypothesised that there would be good agreement between the results of sequential examinations performed on the same slides. If the hypothesis was proven we could be confident that analysis of a single slide on a single occasion would generate results that were a reliable measure of HO-1 and iNOS immunostaining in each sample and could be used in subsequent statistical analysis.

4.3 Methods

Samples of BALF were collected and processed as described in Chapter 2.3. In brief, following sedation with detomidine and butorphanol, BALF was collected using a commercially available BAL tube. Phosphate buffered saline in 6 x 50ml aliquots was instilled as rapidly as possible into the distal airways. The saline was then withdrawn via the tube and the samples in each syringe pooled to give a single sample. Aliquots of 2ml were obtained from the pooled sample and 6 slides were made from one single aliquot within 4 hours of collection. For the purposes of this study only samples that were processed without freezing were used. The results of Chapter 3 indicated that the cytological appearance and immunostaining of nucleated cells was very poor for BALF samples that had been frozen after collection. Immunostained frozen cells were not suitable for use in further investigations. The slides made from fresh samples of BALF were stained for iNOS and HO-1 using rabbit polyclonal antibodies utilizing a Dako EnVision kit K4011 (Dako, Ely, UK) as per manufacturer's instructions and all incubations were performed at room temperature as outlined in Chapter 2. Stained BALF from 14 horses was examined as described in Chapter 2. The quality of staining in isolated samples was very poor and these slides were excluded from analysis. Intra-operator agreement was determined for the 12 sets of slides stained for iNOS and 13 sets of slides stained for HO-1.

Slides were examined by the author who was blinded to the identity of each slide. During examination of each slide, the degree of staining was determined for each cell as described in Chapter 2. Representative regions of each slide were selected and images captured using a digital camera connected to the microscope. One hundred cells were examined for each slide. If insufficient cells were present in a single image capture then further captures were taken from the same slide and if necessary, from additional slides made from the same aliquot until in excess of 100 cells were present or all cells on the slides had been examined. All cells on a capture image were counted to ensure that the count was objective and representative. Cells were classified as neutrophils, macrophages or lymphocytes. Pilot data had indicated that staining of mast cells and eosinophils was inconsistent and that these cell types were difficult to identify with confidence following immunostaining and these cell types were not included in the counts. This process was repeated twice on subsequent days, again with the operator blinded to the identity of each slide. Results were tabulated and displayed graphically.

For each of macrophage, neutrophil and lymphocyte cell types, total numbers of cells staining each grade were calculated as a percentage of the total number of each cell type.

Results (percentages) were initially plotted on a histogram to enable comparison of sequential examinations for each of the samples (appendices 4.1 and 4.2).

4.4 Results

Signalment and clinical findings for horses included are shown in Table 4.4.1. Samples were collected from 14 horses and ponies and a number of different breeds were represented. Ten horses were geldings and four were mares. Mean (± standard deviation) age of the animals was 13.8 ± 5.4 years (range; 4 to 21 years). Eight of the animals were diagnosed with RAO, one with IAD and the remaining 5 were not considered to have respiratory disease. The results of immunocytochemical staining for HO-1 and iNOS are presented in Tables 4.4.2 and 4.4.3 respectively. From these results means and standard deviations were calculated for each cell type for each degree of staining from each horse (Tables 4.4.4 and 4.4.5). Means and standard deviations are displayed graphically in figures 4.4.1 and 4.4.2. The means could be compared to the error bars representing a single standard deviation.

Further consideration was given to more objective analysis of the results. It was not appropriate to calculate coefficients of variation (CV) as several of the means approach or are zero. In these cases CV becomes an unreliable measure of repeatability (Parkin, T.D.H., personal communication). There is also an obvious lack of independence between measures i.e. if the percentage of cells staining 1 was very high it is not possible for the percentage of cells staining 2 and 3 to also be high. ANOVA is not appropriate as data are not normally distributed, nor are the variances for each comparative group equal.

Bland Altman plots were considered as a means of indicating how much agreement is present and it is possible to calculate the 95% confidence intervals for the repeated measures. However, it is then open to clinical judgment as to whether these 95% confidence intervals are so wide as to imply poor repeatability. As with any statistical analysis sample size calculations could be performed to provide an indication of the likely power of the analysis (Bland and Altman 1996a,b,c). The appropriate method as outlined by Bland and Altman (2010) indicates that in order to estimate the true repeatability to within 10% either side of the estimate we would have required either 16 repeated

measurements from the 13 horses or 128 horses each being measured 3 times. Neither scenario was feasible given the time and resource constraints of the study.

Table 4.4.1 Signalment and clinical data for 14 horses from which BALF was collected and prepared slides underwent immunostaining.

Horse	Age	Breed	Gender	Diagnosis
1	18	Standardbred	Gelding	Clinically normal
2	21	Thoroughbred	Mare	RAO
3	11	Cross bred	Gelding	RAO
4	4	Thoroughbred	Gelding	Orthopaedic dz
5	6	Pony	Mare	Clinically normal
6	18	ThoroughbredX	Mare	Clinically normal
7	14	Welsh Se D	Mare	RAO
8	13	Cob	Gelding	IAD
9	9	We Se B	Mare	RAO
10	9	We Se B	Mare	RAO
11	15	Thoroughbred	Mare	Clinically normal
12	21	Pony	Mare	RAO
13	18	Thoroughbred	Mare	RAO
14	16	Pony	Mare	RAO

Table 4.4.2 Haem oxygenase-1 staining for 13 samples of BALF repeated on 3 occasions. The number of cells with each grade of staining grade are calculated as percentages of the total number of each cell type. Ex = examination.

	Grade 0 Staining			Grade 1 Staining			Grade 2 Staining			Grade 3 Staining		
Horse number	Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3
					Macrophages (% of cells staining each grade at each examination)							
1	3	2	21	33	57	75	61	38	4	3	2	0
2	0	5	0	22	5	17	53	76	50	24	14	33
3	10	8	50	5	8	25	10	46	0	76	38	25
4	14	13	21	14	5	35	71	74	38	2	8	6
5	26	20	15	60	49	35	15	17	45	0	14	5
6	0	21	10	55	37	40	40	37	35	4	5	15
7	0	0	11	0	10	6	42	10	72	58	80	11
8	0	23	7	33	28	37	37	21	50	30	28	7
9	40	36	40	45	31	30	9	33	10	5	0	20
10	0	13	0	0	35	54	100	41	46	0	11	0
11	0	0	0	40	40	29	53	50	71	7	10	0
12	0	17	0	11	33	41	60	30	28	29	20	31
13	0	40	14	50	20	57	50	40	14	0	0	14
					Neutrophils (% of cells staining each grade at each examination)							
1	0	8	3	42	45	34	58	32	56	0	16	6
2	50	25	0	50	25	100	0	25	0	0	25	0
3	0	0	0	0	25	0	25	63	100	75	13	0
4	33	0	0	33	100	100	0	0	0	33	0	0
5	0	0	0	38	14	0	63	36	100	0	50	0
6	0	76	50	38	11	50	63	10	0	0	3	0
7	0	7	0	0	7	0	67	33	100	33	53	0
8	0	33	33	80	20	67	20	33	0	0	13	0
9	0	16	20	0	32	40	50	28	20	50	24	20
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	100	0	50	0	100	50	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0
13	4	3	9	50	35	27	46	53	45	0	9	18
					Lymphocytes (% of cells staining each grade at each examination)							
1	0	9	0	40	45	40	60	27	60	0	18	0
2	54	33	54	15	9	15	2	24	2	29	33	29
3	25	24	25	18	13	18	17	50	17	39	12	39
4	69	53	69	22	27	22	9	15	9	0	5	0
5	28	4	28	33	96	33	39	0	39	0	0	0
6	79	51	79	21	49	21	0	0	0	0	0	0
7	100	20	100	0	80	0	0	0	0	0	0	0
8	66	29	66	34	45	34	0	26	0	0	0	0
9	45	8	45	55	33	55	0	42	0	0	17	0
10	0	12	0	68	29	68	32	24	32	0	35	0
11	93	42	93	7	33	7	0	25	0	0	0	0
12	45	90	45	38	10	38	17	0	17	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0

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Table 4.4.3 Inducible nitric oxide synthase staining for 12 samples of BALF repeated on 3 occasions. The number of cells with each grade of staining grade are calculated as percentages of the total number of each cell type.

	Grade 0 Staining			Grade 1 Staining			Grade 2 Staining			Grade 3 Staining		
Horse number	Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3	Ex 1	Ex 2	Ex 3
				Macrophages (% of cells staining each grade at each examination)								
2	0	0	0	31	27	56	58	55	33	11	18	11
3	0	7	0	3	3	4	8	23	4	89	67	92
4	0	0	0	6	4	4	94	95	96	0	1	0
5	32	44	40	68	56	55	0	0	5	0	0	0
7	0	2	3	52	47	23	40	34	72	8	17	2
8	0	0	3	12	18	34	72	77	53	16	5	10
9	13	21	13	33	10	51	53	67	34	0	2	2
10	0	0	8	23	22	40	69	73	33	8	5	19
11	0	0	2	59	60	30	16	10	33	25	29	35
12	0	4	0	60	73	56	31	22	27	9	0	16
13	0	0	0	52	48	14	48	52	86	0	0	0
14	0	0	0	56	68	25	44	32	75	0	0	0
				Neutrophils (% of cells staining each grade at each examination)								
2	0	0	0	100	64	47	0	27	51	0	9	2
3	0	12	0	61	35	29	28	24	38	11	29	33
4	0	20	0	100	20	0	0	60	0	0	0	0
5	0	17	40	100	33	0	0	33	0	0	17	0
7	0	0	3	100	43	36	0	57	64	0	0	0
8	0	0	3	83	76	55	17	24	27	0	0	0
9	7	2	13	25	24	26	68	74	67	0	0	0
10	0	3	8	0	34	53	0	61	38	0	1	7
11	57	63	2	43	25	45	0	13	27	0	0	18
12	0	0	0	0	0	0	0	0	0	0	0	0
13	53	48	0	21	24	60	26	28	20	0	0	0
14	0	0	0	49	0	0	51	0	0	0	0	0
				Lymphocytes (% of cells staining each grade at each examination)								
2	88	75	52	0	25	26	12	0	22	0	0	0
3	11	7	6	22	37	14	23	30	50	45	26	31
4	54	50	48	46	50	40	0	0	13	0	0	0
5	23	0	75	77	100	25	0	0	0	0	0	0
7	81	91	33	19	9	54	0	0	13	0	0	0
8	22	14	21	78	86	50	0	0	29	0	0	0
9	100	60	71	0	10	18	0	30	12	0	0	0
10	0	0	0	50	38	77	50	63	23	0	0	0
11	82	93	85	18	7	5	0	0	5	0	0	5
12	9	6	48	44	42	15	48	52	36	0	0	0
13	0	97	83	0	1	8	0	2	8	0	0	0
14	0	67	100	0	0	0	0	33	0	0	0	0

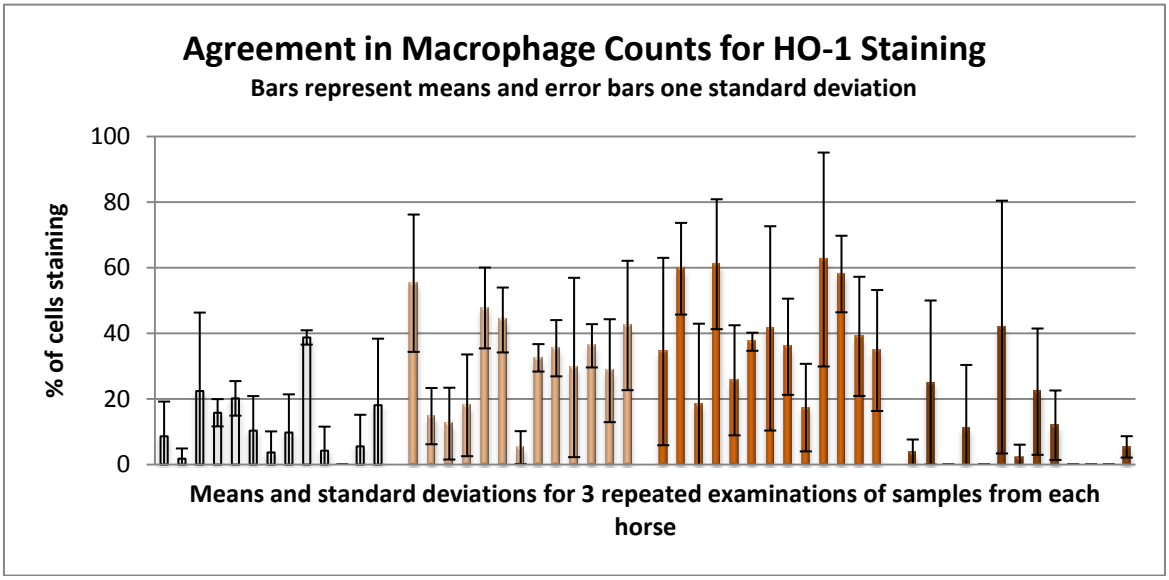
Table 4.4.4 Means and standard deviations data for repeated examination of slides immunostained for HO-1.

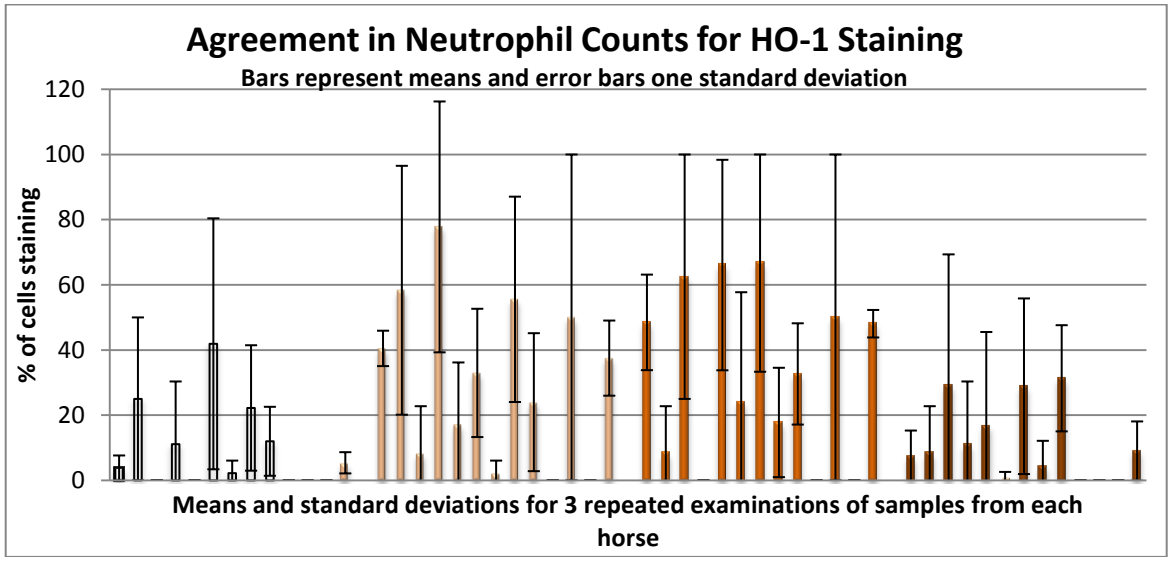
	Grade 0		Grade 1		Grade 2		Grade 3	
Horse	mean	sd	mean	sd	mean	sd	mean	sd
				Macrophages				
1	9	11	55	21	34	29	4	4
2	2	3	15	9	60	14	25	25
3	22	24	12	11	19	24	0	0
4	16	4	18	15	61	20	11	19
5	20	5	48	12	26	17	0	0
6	10	11	44	10	37	3	42	39
7	4	6	5	5	41	31	2	4
8	10	12	33	4	36	15	22	19
9	39	2	35	9	17	13	12	11
10	4	7	30	27	62	33	0	0
11	0	0	36	7	58	12	0	0
12	6	10	29	16	39	18	0	0
13	18	20	42	20	35	18	5	3
				Neutrophils				
1	4	4	40	5	48	15	7	8
2	25	25	58	38	8	14	8	14
3	0	0	8	14	63	38	29	40
4	11	19	78	38	0	0	11	19
5	0	0	17	19	66	32	17	29
6	42	39	33	20	24	34	1	2
7	2	4	2	4	67	33	29	27
8	22	19	56	32	18	17	4	8
9	12	11	24	21	33	16	31	16
10	0	0	0	0	0	0	0	0
11	0	0	50	50	50	50	0	0
12	0	0	0	0	0	0	0	0
13	5	3	38	12	48	4	9	9
				Lymphocytes				
1	3	5	42	3	49	19	6	10
2	47	12	13	4	9	13	30	3
3	25	0	17	3	28	19	30	16
4	64	9	24	3	11	3	2	3
5	20	14	54	36	26	22	0	0
6	69	16	31	16	0	0	0	0
7	73	46	27	46	0	0	0	0
8	53	21	38	6	9	15	0	0
9	33	21	47	12	14	24	6	10
10	4	7	55	23	29	5	12	20
11	76	30	15	15	8	14	0	0
12	60	26	28	16	11	10	0	0
13	0	0	0	0	0	0	0	0

Table 4.4.5 Means and standard deviations data for repeated examination of slides immunostained for iNOS.

	Grade 0		Grade 1		Grade 2		Grade 3	
Horse	mean	sd	mean	sd	mean	sd	mean	sd
				Macrophages				
1	0	0	38	15	49	13	13	4
2	2	4	3	1	12	10	83	14
3	0	0	5	1	95	1	0	1
4	39	6	60	7	2	3	0	0
5	2	2	40	15	49	20	9	8
6	1	2	22	11	67	12	10	6
7	16	5	31	21	51	16	2	1
8	3	4	28	11	58	22	11	7
9	1	1	50	17	20	12	30	5
10	1	3	63	9	27	4	8	8
11	0	0	38	21	62	21	0	0
12	0	0	50	22	50	22	0	0
				Neutrophils				
1	0	0	70	27	26	25	4	5
2	4	7	42	17	30	7	25	12
3	7	12	40	53	20	35	0	0
4	19	20	44	51	11	19	6	10
5	1	2	60	35	40	35	0	0
6	1	2	71	15	23	5	0	0
7	7	5	25	1	69	4	0	0
8	4	4	29	27	33	31	3	4
9	41	33	38	11	13	14	6	10
10	0	0	0	0	0	0	0	0
11	34	29	35	22	25	4	0	0
12	0	0	16	29	17	29	0	0
				Lymphocytes				
1	72	18	17	15	11	11	0	0
2	8	3	24	12	34	14	34	10
3	50	3	45	5	4	7	0	0
4	33	38	67	38	0	0	0	0
5	69	31	27	24	4	7	0	0
6	19	4	71	19	10	16	0	0
7	77	21	9	9	14	15	0	0
8	0	0	55	20	45	20	0	0
9	87	6	10	7	2	3	2	3
10	21	24	34	16	45	8	0	0
11	60	52	3	5	4	4	0	0
12	56	51	0	0	11	19	0	0

Figure 4.4.1. Means and standard deviations for cells immunostained for HO-1. Each graph represents a different cell type and each bar a different horse. The grade of staining is indicated by the colour of the bar; grade 0 (clear) on the left through to grade 3 (dark orange) on the right.





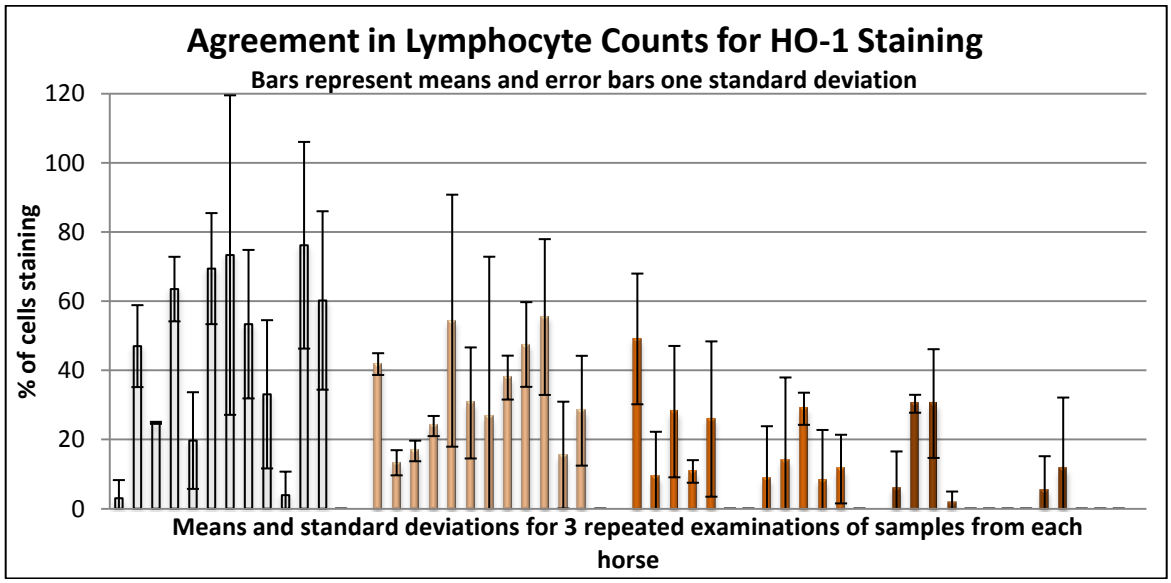
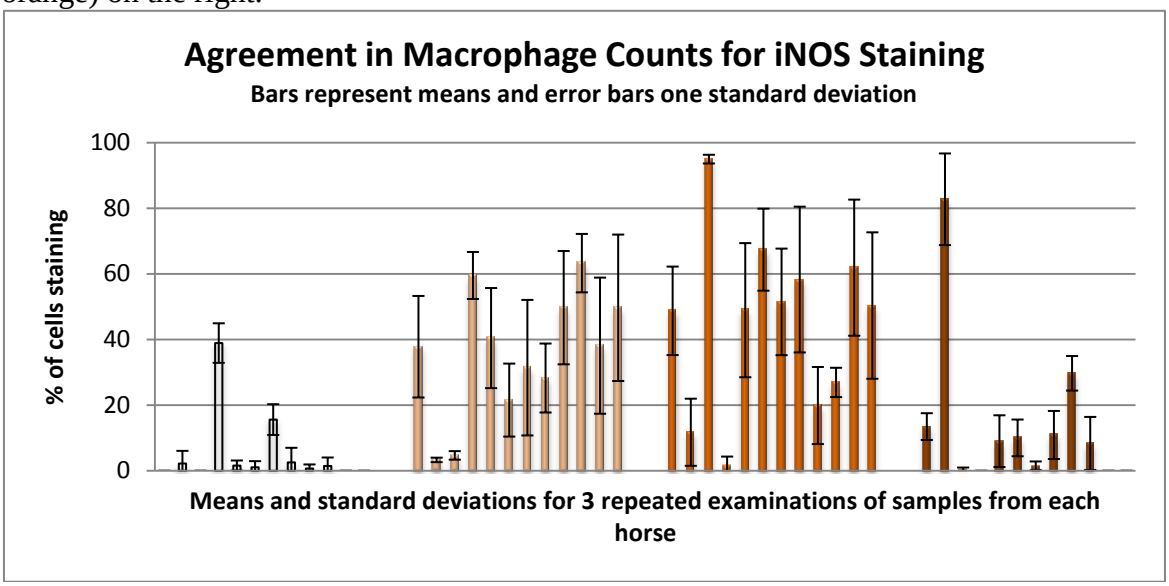
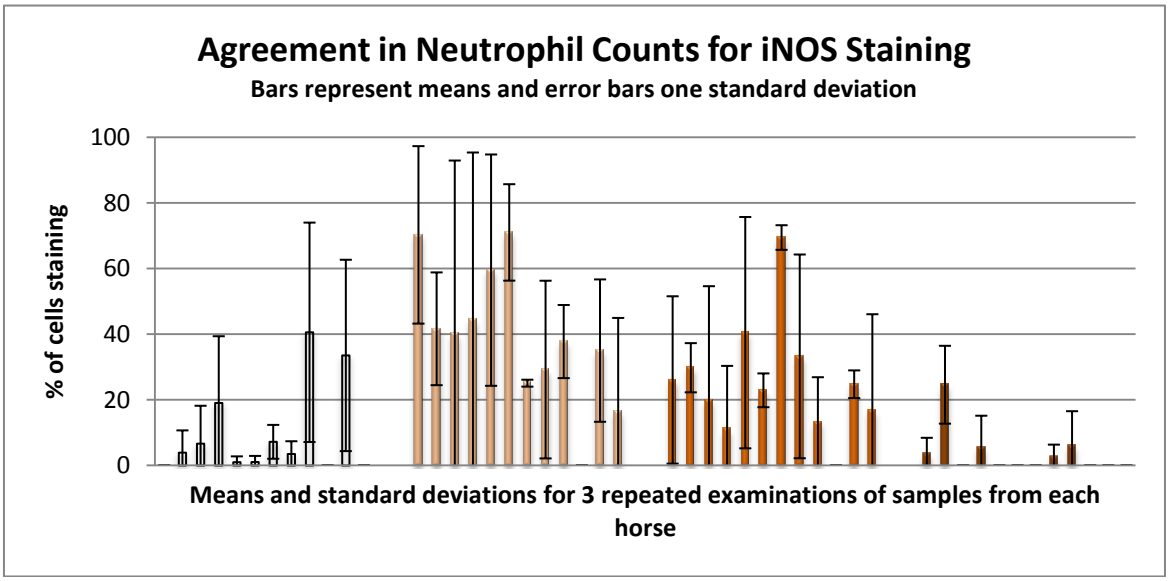
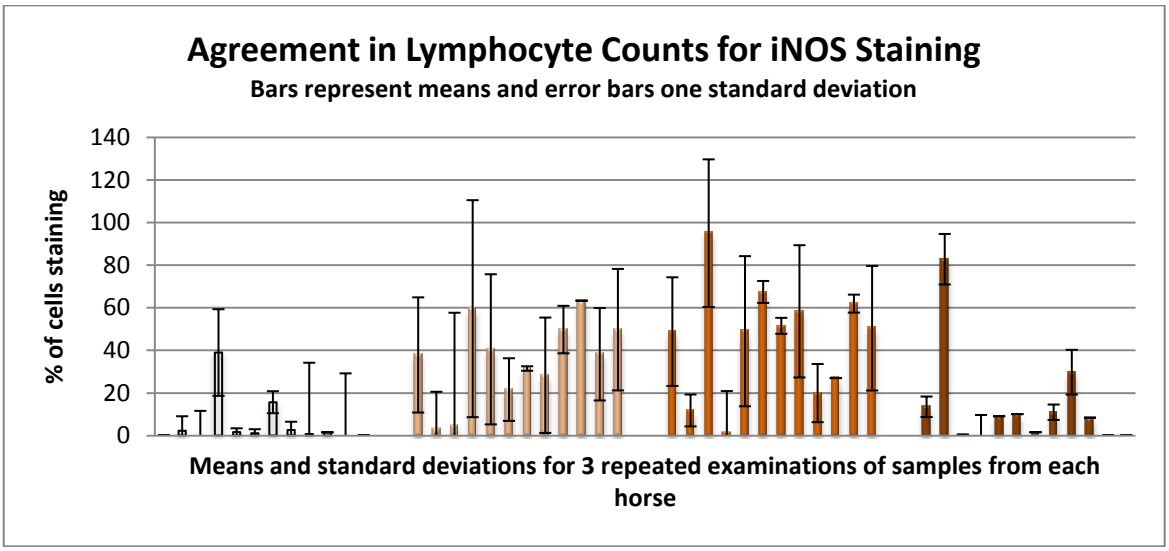


Figure 4.4.2. Means and standard deviations for cells immunostained for iNOS. Each graph represents a different cell type and each bar a different horse. The grade of staining is indicated by the colour of the bar; grade 0 (clear) on the left through to grade 3 (dark orange) on the right.







4.5 Discussion

Given the limited number of horses and samples measured per horse it is difficult to reach a definitive conclusion on the repeatability of this method for assessing immunostaining of leukocytes in equine BALF. The results do show that we would require a large number of horses or sample measurements to reach a conclusion that had sufficient statistical power to indicate the likely true repeatability of this assessment. Nevertheless as only a single operator was used for all measurements (thus removing any inter-operator error) and taking account of time and resource restrictions, it was felt appropriate to continue to use this method of subjective analysis throughout the rest of the study. The results obtained in subsequent chapters are thus limited by our failure to demonstrate definitively that methods were repeatable. The conclusions drawn in subsequent chapters are done so with this caveat in mind and furthermore being cognicant of the fact that subsequent evaluations were based upon a single observation of staining for each sample.

The standard deviation from the mean was greater overall for neutrophils than for other cell types. The reason why neutrophil counts were more variable is uncertain. There did not appear to be any differences in repeatability between staining for iNOS and HO-1.

Further analysis could be performed to determine whether results are reproducible between observers. In subsequent chapters of the current study all the analyses were performed by a single observer but should more observers be required then inter-observer repeatability ought to be assessed. A further limitation of this study was the failure to establish whether the methods used to prepare the slides are repeatable. The same slides were used in this study with no investigation of potential differences that might occur between slides. Different slides prepared from the same sample were compared during the initial analysis and although an impression was gained that the slides were similar this was not tested objectively. Case numbers were limited and the majority of cases were affected by one disease condition, namely RAO. Ideally, a greater number of samples would have been analysed in this study and the samples would have been collected from normal animals as well as from animals with a range of respiratory diseases. It is possible that the degree of repeatability may be influenced by degrees and/or patterns of staining.

CHAPTER FIVE

EXPRESSION OF H0-1 WITHIN THE INFLAMMATORY CELLS OF EQUINE BALF

5.1 Introduction

Evidence for a role of HO-1 activity has been identified in the pathogenesis of numerous pulmonary diseases in man including acute respiratory distress syndrome (ARDS), interstitial pulmonary fibrosis, COPD and asthma. Asthma is a chronic inflammatory airway disease associated with increased ROS and inflammatory mediators, all of which have been demonstrated to induce expression of HO-1 (Barnes 1990). The pathogenesis of RAO has some parallels with the pathogenesis of asthma and oxidative stress has been identified in affected animals during exacerbation of disease (Art *et al.* 1999; Deaton *et al.* 2004a; Deaton *et al.* 2004b; Deaton *et al.* 2006). Haem oxygenase-1 is induced in airways during asthma (Carter *et al.* 2004) and it is possible that it will be similarly induced in the airways of horses with lower airway inflammation (Wyse *et al.* 2006).

In human airways, the distribution of HO-1 has been investigated using immunostaining and western blot techniques. Immunostaining has identified HO-1 to be distributed throughout the submucosa of the distal airways, particularly the airway epithelium and submucosal macrophages (Lim *et al.* 2000; Maestrelli *et al.* 2001). Further evidence for macrophages being the predominant source of HO-1 was provided in a mouse model of asthma with immunofluoresecence occurring mostly in the cytoplasm and particularly in the perinuclear region (Maestrelli *et al.* 2001). In a model of inflammation in the pleural cavity, western blot analysis revealed an increase in HO-I protein in macrophages but not in neutrophils (Minamino *et al.* 2001). Haem oxygenase-1 expression has been demonstrated in alveolar macrophages, neutrophils and type II epithelial cells from normal equine lungs (Wyse *et al.* 2006).

5.2 Aims

The aims of this study where to determine whether there are relationships between HO-1 staining in leukocytes from equine BALF and other markers of respiratory tract inflammation. It was hypothesised that there would be positive correlation between the degree of HO-1 staining in leukocytes from BALF and recognised markers of lower airway inflammation.

5.3 Methods

Horses presented for the investigation of respiratory disease or poor performance were examined and clinical findings recorded as outlined in Chapter 2.2. Samples of BALF were collected and processed as described in Chapter 2.3. In brief, following sedation, BALF was collected using a commercially available BAL tube. Phosphate buffered saline was instilled into the distal airways and then aspirated via the tube. The retrieved fluid was pooled to give a single sample. Slides were prepared from each BALF sample for immunostaining within 4 hours of collection. The slides were stained for HO-1 using rabbit polyclonal antibodies utilizing a Dako EnVision kit K4011 (Dako, Ely, UK) as per manufacturer's instructions and all incubations were performed at room temperature as outlined in Chapter 2. Stained slides prepared from BALF from 13 horses were examined and the degree of staining determined for a representative sample of cells as described in Chapter 2.6. The results of cytological examinations for one horse were lost and comparisons of immunostaining and cytological analysis were therefore performed for 12, rather than 13, horses. Staining was graded on a 0-3 scale for each cell.

From the results of examination of immunocytochemical staining of cells, the following calculations were made:

i) the percentage of cells with any stain uptake (i.e. grades 1-3)

ii) the percentage of cells staining greater than 1 (i.e. grades 2 and 3)

iii) the percentage of cells staining greater than 2 (i.e. grade 3).

Pearson's correlation was used to compare the continuous immunocytochemical staining data with continuous clinical and cytological data. Mann-Whitney tests were performed to compare the categorical immunocytochemical staining data with categorical clinical data.

For the cough, mucus and clinical score categories, horses with no abnormalities (no cough, no mucus and a clinical score of 0) were compared to horses with abnormal results.

Possible associations between HO-1 immunostaining of leukocytes in BALF and lower airway inflammation was investigated further by comparing staining in horses considered to have neutrophilic airway inflammation with those considered to have normal airways. Horses were divided into 2 groups: horses with a neutrophil relative percentage of > 5% in BALF were considered to have lower airway inflammation and horses with < 5% neutrophils were classified as free of lower airway inflammation. This single indicator of airway inflammation was chosen as it is the most reliable indicator of lower airway inflammation (Robinson 2001). The degree of HO-1 immunostaining of inflammatory cells in BALF in the diseased and control groups was compared using a Mann-Whitney test.

5.4 Results

There were nine horses with neutrophilic lower airway disease and three horses that were considered to have no cytological evidence of lower airway inflammation. The result of cytological analysis of BALF for one horse (horse 4) was lost. The results of clinical examinations, cytological examinations and immunostaining are presented in table 5.4.1. Eight horses had a history of coughing and were compared to 5 that did not and seven horses in which mucus had been identified were compared with 6 in which it was not. For both clinical score and neutrophilic inflammation, 10 horses classified as having abnormal results were compared to 3 that were considered normal. Neutrophils were more likely to stain for HO-1 than other cell types. Results of statistical analysis are presented in Table 5.4.2. Statistically significant associations were identified between the percentage of macrophages staining >1 and mast cell percentages; between the percentage of macrophages staining >2 and both respiratory rate and neutrophil percentage. In addition the percentage of neutrophils staining greater than 1 or 2 was associated with respiratory rate and clinical score. The percentage of lymphocytes staining greater than 1 or 2 was associated with eosinophil percentage.

Figure 5.4.1. Leukocytes from equine BALF stained for HO-1

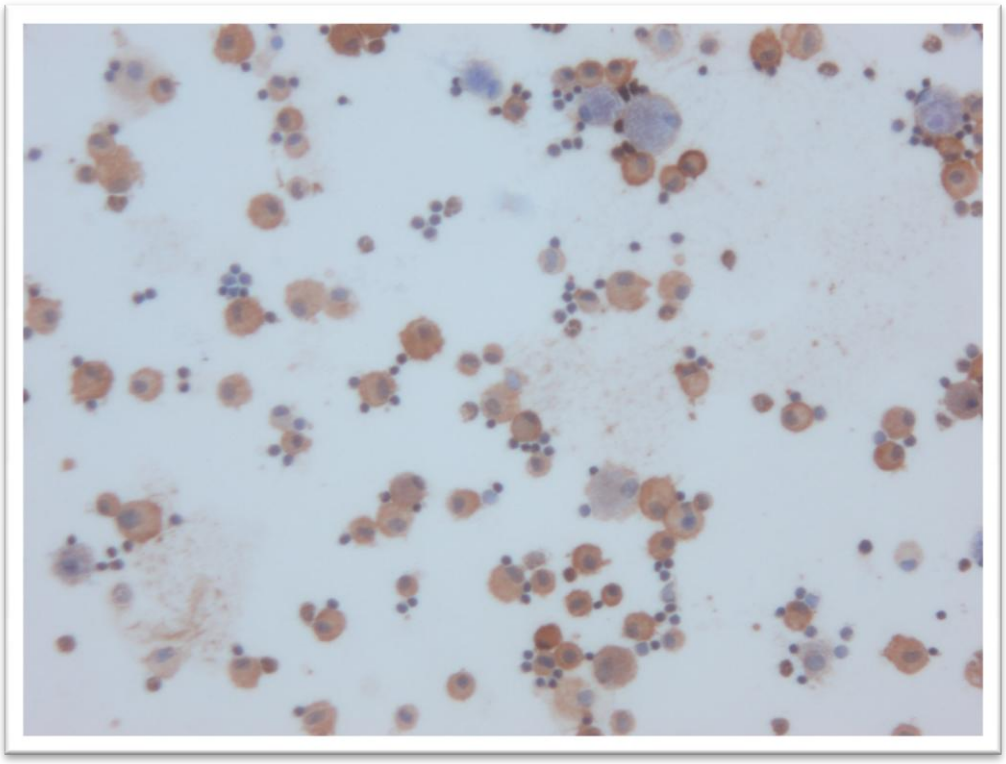


Table 5.4.1 Clinical and cytology findings and HO-1 immunostaining results for 13 cases of suspected respiratory disease or poor performance. Respiratory rate (Resp), neutrophil (Neut), macrophage (Macro), lymphocyte (Lymp), eosinophil (Eos) and mast cell (Mast).

	Cough	Resp (breaths per minute)	Mucus grade	Clinical score	BALF cytology					Macrophages			H
					Neut (%)	Macro (%)	Lymph (%)	Eos (%)	Mast (%)	%>0	%>1	%>2	%>
1	Yes	8	1	0	3	49	39	2	7	97.1	63.8	2.9	100
2	Yes	16	0	0	11	33	45	0	11	100.0	77.8	24.4	50.
3	Yes	32	3	2	29	26	36	3	6	90.5	85.7	76.2	100
4	No	12	0	0						86.4	72.7	1.5	66.
5	Yes	16	1	0	0	100	0	0	0	74.5	14.9	0.0	100
6	Yes	16	0	0	4	35	56	0	5	100.0	44.7	4.3	100
7	Yes	36	2	4	72	15	11	0	2	100.0	100.0	57.7	100
8	No	16	0	0	27	69	3	0	1	100.0	67.4	30.4	100
9	No	28	2	4	23	64	11	0	2	60.0	14.7	5.3	100
10	No	16	1	0	22	62	6	0	10	100.0	100.0	0.0	100
11	Yes	16	0	0	15	30	50	0	5	100.0	60.0	7.3	100
12	Yes	16	0	0	13	41	35	0	11	100.0	88.9	28.9	100
13	No	16	0	0	18	23	55	0	5	100.0	50.0	0.0	95.

Table 5.4.2. Results of Pearson's correlations used to investigate possible assocations between the degree of staining for HO-1 and respiratory rate (Resp), neutrophil (Neut) %, macrophage (Macro) %, lymphocyte (Lymp) %, eosinophil (Eos) % and mast cell (Mast) %. Significant results ($p<0.05$) are highlighted in bold.

		Resp	Neut %	Macro %	Lymp %	Eos %	Mast %
% macrophages staining >0 for H0-1	r value	-0.248	0.124	-0.563	0.448	-0.013	0.489
	p value	0.414	0.701	0.057	0.144	0.967	0.107
% macrophages staining >1 for HO-1	r value	0.155	0.504	-0.533	0.044	0.203	0.580
	p value	0.614	0.094	0.075	0.893	0.527	**0.048**
% macrophages staining >2 for HO-1	r value	0.736	0.651	-0.453	-0.087	0.493	-0.008
	p value	**0.004**	**0.022**	0.139	0.787	0.103	0.981
% neutrophils staining >0 for HO-1	r value	0.244	0.148	0.188	-0.271	0.143	-0.462
	p value	0.422	0.645	0.558	0.394	0.657	0.131
% neutrophils staining >1 for HO-1	r value	0.684	0.376	-0.101	-0.158	0.393	-0.452
	p value	**0.010**	0.228	0.756	0.623	0.206	0.141
% neutrophils staining >2 for HO-1	r value	0.744	0.489	-0.230	-0.161	0.569	-0.226
	p value	**0.004**	0.107	0.472	0.617	0.053	0.480
%lymphocytes staining >0 for HO-1	r value	-0.298	-0.416	0.300	-0.037	0.382	0.298
	p value	0.322	0.178	0.344	0.909	0.221	0.346
% lymphocytes staining >1 for HO-1	r value	-0.177	-0.343	0.085	0.118	0.716	0.333
	p value	0.562	0.275	0.794	0.715	**0.009**	0.290
% lymphocytes staining >2 for HO-1	r value	0.351	0.040	-0.315	0.236	0.609	0.326
	p value	0.239	0.902	0.318	0.461	**0.036**	0.300

Table 5.4.3. Results of Mann-Whitney tests used to investigate possible associations between the degree of staining for HO-1 and coughing, mucus score, clinical score and neutrophilic inflammation. Significant results ($p<0.05$) are highlighted in bold.

		Cough	Mucus	Clinical Score	Neutrophilic Inflammation
% macrophages staining >0 for HO-1	w value	42.000	34.000	16.000	71.500
	p value	0.626	0.871	0.579	0.159
% macrophages staining >1 for HO-1	w value	32.500	47.000	67.500	72.000
	p value	0.770	0.830	0.735	0.190
% macrophages staining >2 for HO-1	w value	26.000	50.000	59.000	74.000
	p value	0.211	0.943	0.074	0.103
% neutrophils staining >0 for HO-1	w value	30.500	42.000	22.000	63.500
	p value	0.428	0.257	0.918	1.000
% neutrophils staining >1 for HO-1	w value	29.000	35.500	36.000	32.000
	p value	0.415	0.060	**0.014**	0.585
% neutrophils staining >2 for HO-1	w value	37.500	40.500	55.500	65.500
	p value	0.720	0.162	**0.004**	0.706
% lymphocytes staining >0 for HO-1	w value	40.000	40.000	73.000	28.000
	p value	0.508	0.222	0.671	1.000
%lymphocytes staining >1 for HO-1	w value	31.000	40.000	64.500	33.000
	p value	0.608	0.212	0.391	0.475
% lymphocytes staining >2 for HO-1	w value	33.000	48.000	66.000	63.500
	p value	0.767	0.943	0.347	1.000

5.5 Discussion

In the current study the percentage of neutrophils staining greater than 1 and greater than 2 for HO-1 correlated with both respiratory rate and clinical score. However, there was no correlation between the total number of neutrophils staining for HO-1 and either of these clinical parameters. When assessing equine respiratory disease the number of neutrophils as a percentage of the total number of leukocytes in BALF is considered a reliable means of assessing the severity and correlates with histopathological changes in RAO (Larson and Busch 1985). It was therefore surprising that whilst HO-1 expression appeared to be related to clinical indicators of respiratory disease there was no relationship with relative neutrophilia. The significant associations that were identified between the percentage of neutrophils staining greater than 1 and 2 and clinical score are of questionable clinical relevance as only 3 horses actually had an abnormal clinical score and hence analysis was based upon very small numbers. Further investigations with more horses demonstrating more marked evidence of respiratory dysfunction at rest ought to be performed. No statistically significant associations were identified between HO-1 expression and the presence of a cough or tracheal mucus.

Other associations between immunostaining of specific leukocyte types in BALF and clinical findings of respiratory disease were found also. The percentage of macrophages staining greater than 1 for HO-1 correlated with the percentage of mast cells in BALF however this result was barely significant ($p=0.048$). The percentage of macrophages staining greater than 2 for HO-1 correlated with neutrophil percentage in BALF and with respiratory rate. The percentage of lymphocytes staining greater than 1 and greater than 2 for HO-1 correlated with the percentage of eosinophils in BALF. Correlations with BALF eosinophil ratio are of questionable relevance as eosinophils were only identified in 2 horses and results are therefore based upon very limited data. Similarly lymphocytes only stained grade 3 in 2 horses making associations with this variable equally equivocal.

Although statistically significant associations between degrees of HO-1 staining in leukocytes in BALF and indicators of respiratory disease were sporadic, it is plausible that there is an association between HO-1 expression in neutrophils and macrophages and equine lower airway disease. Similar associations have been identified in human respiratory disease. Haem oxygenase-1 is induced in airways during asthma (Carter *et al.* 2004) and levels of HO-1 expression in macrophages from sputum have been demonstrated to be increased in asthmatic patients compared to healthy controls (Horvath *et al.* 1998). In another study comparing smoking and non-smoking human subjects,

immunostaining was identified predominantly in alveolar macrophages (a median of 36% staining positive in smokers and 13% in nonsmoking subjects) (Maestrelli *et al.* 2001).

Inflammation, infection, sepsis and endotoxaemia all result in ROS production and in these conditions HO-1 induction may be protective via the biological roles of its products (section 1.3.4) (Rizzardini *et al.* 1993; Rizzardini *et al.* 1994). Numerous cytoprotective functions for HO-1 and its products have been identified. There is evidence that HO-1 is cytoprotective in the face of oxidative stress, lung apoptosis and inflammation (Keyse and Tyrell 1987, 1989; Otterbein et al. 1999a; Christou *et al.* 2000; Minamino et al. 2001). Haem oxygenase-1 activity decreases inflammatory cell rolling, adhesion and migration from the vascular compartment (Wagener *et al.* 2001). Bilirubin has anti-oxidant activity, inhibits leukocyte rolling, stabilises mast cell membranes and decreases TGF-β expression (Hayashi et al 1999; Wang et al 2002). Carbon monoxide protects against inflammatory hyperoxic lung injury by down-regulation of the expression of pro-inflammatory cytokines. In a model of hyperoxia-induced lung injury the presence of CO prolonged survival, likely as a result of its anti-inflammatory actions (Ryter *et al.* 2002). Carbon monoxide inhibited the appearance of markers of lung injury (haemorrhage, fibrin deposition, oedema, protein accumulation in the airway) and oxidative damage (lipid peroxidation) (Ryter *et al.* 2002). Carbon monoxide also inhibited the influx of neutrophils in BALF (Ryter *et al.* 2002). Carbon monoxide production correlated with inhibited expression of the pro-inflammatory cytokines IL-1β, TNFα and IL-6 (Ryter *et al.* 2002).

The stage in the disease process at which BALF samples were collected may have influenced the degree of HO-1 expression. In the face of acute inflammation, HO activity initially decreases with the highest levels of expression being recorded as inflammation is resolving (Willis *et al.* 1996). Willis *et al.* (1996) speculated that during tissue inflammation there may be sequential induction of enzymes with pro-inflammatory effects, for example COX-2 and iNOS, followed by increased expression of enzymes with anti-inflammatory effects, including HO-1, which are responsible for the resolution of inflammatory lesions and aimed at preventing a chronic inflammatory response.

The effects of timing on HO-1 expression in horses could be investigated further; however challenge studies in horses known to suffer from RAO would probably be required to ensure sample collection early in the course of the inflammatory response. Although Willis *et al.* 2006 found higher levels of HO-1 expression as inflammation is resolving, it is also possible that cells may become activated and express HO-1 before they reach the airways and are sampled in BALF.

A limitation of the current study is the assumption that expression is likely to correlate with activity. It was also our intention to investigate the activity of HO-1 in sections of pulmonary tissue from horses with respiratory disease and control animals in addition to investigating expression in BALF in order to follow-on from the work of Wyse *et al.* (2006). Wyse *et al.* (2006) found detectable HO-1 activity in both the cranioventral and caudodorsal lung lobes; with activity being comparable between the 2 regions. The activity of HO-1 has been measured in human lung tissue (Tenhunen *et al.* 1968; Carraway *et al.* 1998). The methods are relatively straightforward and were used in preliminary work in the current study (data not shown). This method was used successfully for measurement of HO-1 activity in the lungs of horses subjected to euthanasia at the University of Glasgow for reasons other than respiratory disease and the results were consistent with those of Wyse *et al.* (2006). It was expected that through the study period a small number of cases with respiratory tract inflammation would be subjected to euthanasia even if respiratory tract disease was not the primary reason for euthanasia. Unfortunately, insufficient horses with respiratory disease were recruited to the study. Further studies of the expression and activity of HO-1 within the respiratory tract during health and disease are required to clarify whether there is an association between HO-1 activity and respiratory disease. These studies ought to use alternative methods of objective assessment of HO-1 staining given the failure in this study to clearly demonstrate that methods of assessment were repeatable.

Considering the number of correlations performed in this study the apparently statistically significant results may have arisen purely by chance. Further investigations ought to be performed with greater numbers of horses in order to substantiate our findings. A limitation of the current study was the lack of samples from horses considered to be free from lower airway disease. Ethical constraints prohibited sampling from normal horses and few of the horses sampled turned out to be free from respiratory disease. The majority of cases included in the current study were suffering from RAO. Ethical constraints also prevented collection of pulmonary or bronchial biopsies from horses in the current study as they are rarely justified clinically. Comparison of patterns of immunostaining in samples of tissue and BALF collected simultaneously would have added useful data.

CHAPTER SIX

EXPRESSION OF iNOS WITHIN INFLAMMATORY CELLS OF EQUINE BALF

6.1 Introduction

Nitric oxide, produced by NOS has been implicated as a mediator of airway inflammation in human asthma and is produced in large quantities from bronchial epithelial and inflammatory cells of affected patients (Barnes and Liew 1995). In humans iNOS was first identified in macrophages (Pechkovsky *et al.* 2002), however it has since been identified within the cytosol of a diverse range of cell types (Fischer *et al.* 2002) including alveolar type II epithelial cells (Warner *et al.* 1995), lung fibroblasts (Romanska *et al.* 2002), airway and vascular smooth muscle cells (Thomae *et al.* 1993; Xue *et al.* 1994; Griffiths *et al.* 1995), bronchial epithelial cells (Adcock *et al.* 1994; Robbins *et al.* 1994; Watkins *et al.* 1997; Pechkovsky *et al.* 2002), mast cells (Gilchrist *et al.* 2002), endothelial cells (Ermert *et al.* 2002), neutrophils (Boucher *et al.* 1999) and chondrocytes (Kobzik *et al.* 1993; Ricciardolo *et al.* 2006).

In rodent models of allergic airway inflammation, iNOS inhibitors have been shown to decrease eosinophil infiltration and cytokine release. Some authors have suggested that iNOS inhibitors may have a therapeutic benefit in cases of allergic airway disease that are refractory to corticosteroid treatment (Hansel *et al.* 2003). Little research has been performed into the expression of NOS and synthesis of NO in the horse. Increased expression of iNOS has been identified in leukocytes within the mucosa and submucosa of the intestine and vasculature of horses with ischaemic intestinal injury (Mirza *et al.* 1999; Mirza *et al.* 2005). Investigation of NOS expression and NO production in equine respiratory disease is limited to the findings of a single study in which 7 horses with SPA-RAO and 6 horses not affected by the disease were compared (Costa *et al.* 2001). Nitric oxide concentrations in plasma, BALF and ELF were determined using a chemiluminescence method whilst iNOS, nitrotyrosine and nicotinamide (NT) adenine

dinucleotide phosphate diaphorase (NADPHd) were localised in formalin-fixed lung specimens using immunohistochemical and histochemical staining.

Nitric oxide produced by the activity of iNOS may be an important mediator in equine lower airway inflammation. Further characterization of the expression of iNOS may assist in the understanding of the potential roles and importance of NO in health and disease of the lower respiratory tract of horses.

6.2 Aims

The aims of this study were to determine whether there is a relationship between iNOS staining in leukocytes from equine BALF and other markers of respiratory tract inflammation. It was hypothesised that there would be positive correlation between the degree of iNOS staining in leukocytes from BALF and markers of lower airway inflammation.

6.3 Methods

Horses presented for the investigation of respiratory disease or poor performance were examined and clinical findings recorded as outlined in Chapter 2.2. Samples of BALF were collected and processed as described in Chapter 2.3. In brief, following sedation, BALF was collected using a commercially available BAL tube. Phosphate buffered saline was instilled into the distal airways, aspirated via the tube and fluid recovered was pooled to give a single sample. Slides were prepared for immunostaining within 4 hours of collection. The slides were stained for HO-1 using rabbit polyclonal antibodies utilizing a Dako EnVision kit K4011 (Dako, Ely, UK) as per manufacturer's instructions with all incubations at room temperature as outlined in Chapter 2. Stained BALF from 12 horses were examined and the degree of staining determined for a representative sample of cells as described in Chapter 2.6. The results of cytological examinations for one horse were lost and comparisons of immunostaining and cytological analysis were therefore performed for 11, rather than 12, horses. Immunocytochemical staining was graded on a 0-3 scale for each cell. From these results the following calculations were made:

i) the percentage of cells with any stain uptake (i.e. grades 1-3)

ii) the percentage of cells staining greater than 1 (i.e. grades 2 and 3)

iii) the percentage of cells staining greater than 2 (i.e. grade 3).

Pearson's correlations were used to compare immunocytochemical staining categorical data with continuous clinical data. Mann-Whitney tests were performed for comparison of immunocytochemical staining data and categorical clinical data. For the cough, mucus and clinical score categories horses with no abnormalities (no cough, no mucus and a clinical score of 0) were compared to horses with abnormal results.

The relationship between NO immunostaining of leukocytes in BALF and lower airway inflammation was investigated further by comparing staining in horses with the degree of neutrophilia in BALF. Horses were divided into 2 groups: horses with a neutrophil relative percentage of > 5% in BALF were considered to have lower airway inflammation and horses with < 5% neutrophils were classified as free of lower airway inflammation. This single indicator of airway inflammation was chosen as it is the most reliable indicator of lower airway inflammation (Robinson 2001). The degree of NO immunostaining of inflammatory cells in BALF in the diseased and control groups was compared using a Mann-Whitney test.

6.4 Results

There were nine horses with neutrophilic lower airway disease and three horses that were considered to have no cytological evidence of lower airway inflammation. The results of cytological analysis of BALF for one horse (horse 4) were lost. Seven horses had a history of coughing and were compared to 5 that did not and seven horses in which mucus had been identified were compared with 5 in which it was not. For clinical score 10 horses were classified as having abnormal results compared to 2 that were considered normal. For neutrophilic inflammation, nine horses were classified as having abnormal results compared to 3 that were considered normal. Significant associations were identified between the number of macrophages staining > 0 for iNOS and the number of macrophages present in BALF as well as the presence of a cough. The number of macrophages and lymphocytes staining greater than 2 were significantly associated with respiratory rate. The number of lymphocytes staining >1 was associated with the percentages of eosinophils and mast cells in BALF. Staining for iNOS of >2 in all cell types was associated with eosinophil percentages.

Figure 6.4.1. Leukocytes from equine BALF stained for iNOS

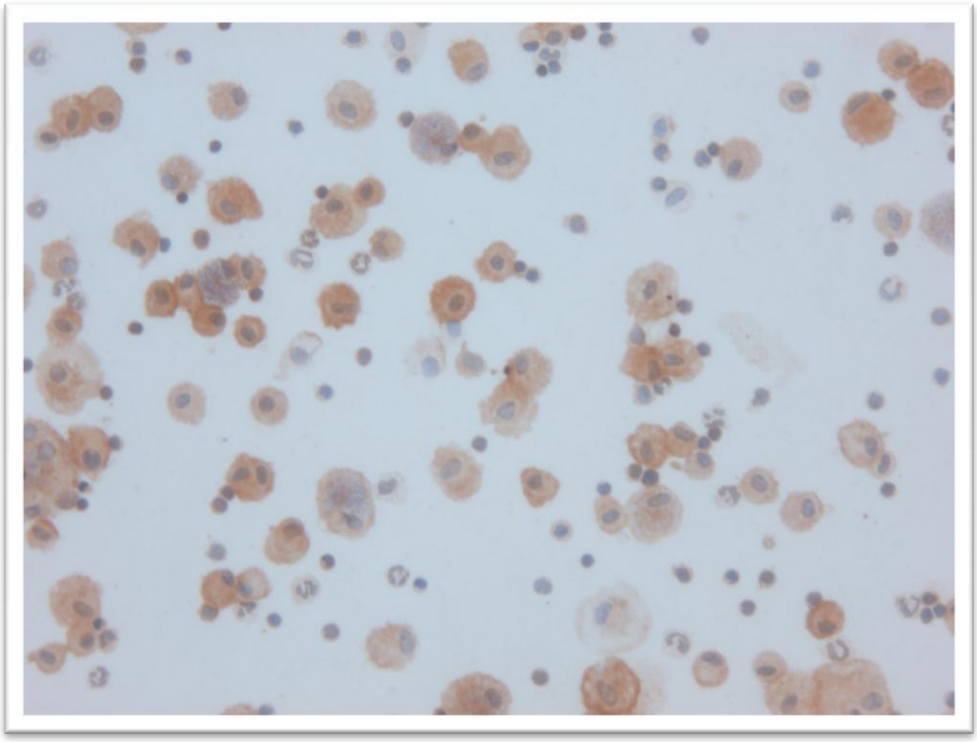


Figure 6.4.2. Neutrophils from equine BALF staining for iNOS

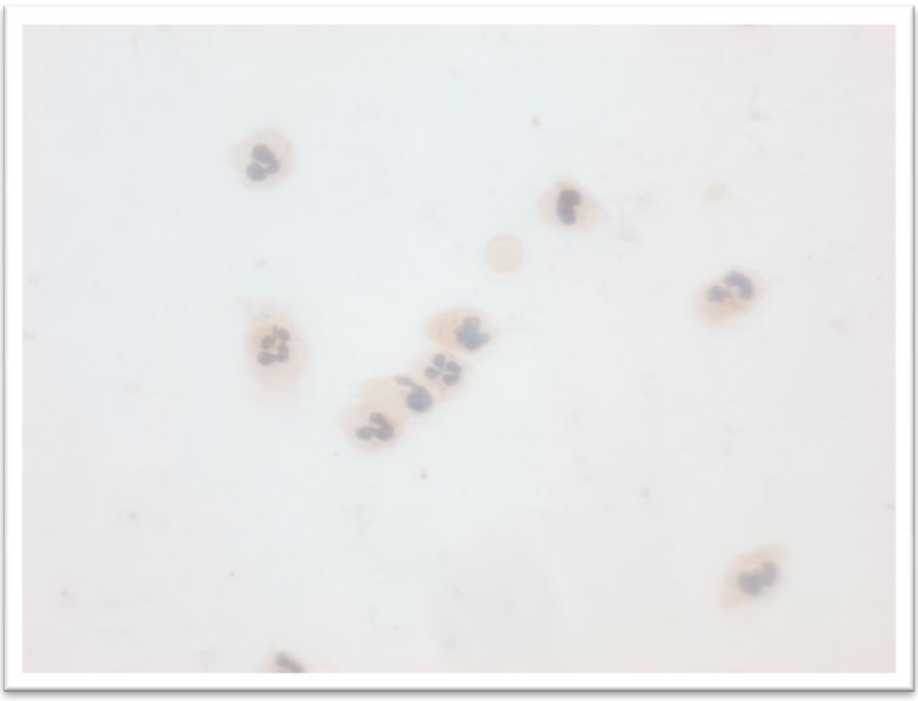


Table 6.4.1 Clinical and cytology findings and iNOS immunostaining results for 12 cases of suspected respiratory disease or poor performance.

	Cough	Respiratory rate (breaths per minute)	Mucus grade	Clinical score	BALF cytology							
					Neut (%)	Macro (%)	Lymph (%)	Eos (%)	Mast (%)	Macrophages %>0	%>1	%>
2	Yes	16	0	0	11	33	45	0	11	100.0	69.4	11.
3	Yes	32	3	2	29	26	36	3	6	100.0	97.4	89.
4	No	12	0	0	0					100.0	93.9	0.0
5	Yes	16	1	0	0	100	0	0	0	67.7	0.0	0.0
6	Yes	16	0	0	4	35	56	0	5	100.0	48.3	8.3
8	No	16	0	0	27	69	3	0	1	100.0	87.7	15.
9	No	28	2	4	23	64	11	0	2	86.7	53.3	0.0
10	No	16	1	0	22	62	6	0	10	100.0	77.4	8.1
11	Yes	16	0	0	15	30	50	0	5	100.0	41.0	24.
12	Yes	16	0	0	13	41	35	0	11	100.0	40.0	8.9
13	No	16	0	0	18	23	55	0	5	100.0	48.0	0.0
14	Yes	24	1	0	89	5	3	0	3	100.0	44.4	0.0

Table 6.4.2. Results of Pearson's correlations comparing the degree of staining for iNOS with respiratory rate (Resp rate), neutrophil (Neut) %, macrophage (Macro) %, lymphocyte (Lymph) %, eosinophil (Eos) % and mast cell (Mast) %. Significant results ($p<0.05$) are highlighted in bold.

		Resp rate	Neut %	Macro %	Lymph %	Eos %	Mast %
% macrophages staining >0 for iNOS	r value	-0.060	0.256	-0.757	0.474	0.135	0.553
	p value	0.853	0.422	**0.007**	0.141	0.691	0.077
% macrophages staining >1 for iNOS	r value	0.168	0.051	-0.271	0.052	0.520	0.307
	p value	0.603	0.874	0.420	0.880	0.101	0.358
% macrophages staining >2 for iNOS	r value	0.599	0.056	-0.245	0.219	0.954	0.126
	p value	**0.040**	0.864	0.467	0.518	**<0.001**	0.712
% neutrophils staining >0 for iNOS	r value	0.163	0.087	0.307	-0.527	0.160	0.074
	p value	0.613	0.789	0.359	0.096	0.638	0.828
% neutrophils staining >1 for iNOS	r value	0.394	0.366	-0.083	-0.287	0.058	0.335
	p value	0.205	0.241	0.808	0.392	0.866	0.314
% neutrophils staining >2 for iNOS	r value	0.576	0.106	-0.103	-0.034	0.874	0.244
	p value	0.050	0.742	0.763	0.921	**<0.001**	0.470
% lymphocytes staining >0 for iNOS	r value	0.024	-0.255	-0.121	-0.332	0.356	0.210
	p value	0.944	0.423	0.724	0.319	0.283	0.536
% lymphocytes staining >1 for iNOS	r value	0.444	0.013	-0.121	0.055	0.665	0.619
	p value	0.171	0.969	0.724	0.872	**0.026**	**0.042**
% lymphocytes staining >2 for iNOS	r value	0.754	0.105	-0.251	0.161	0.997	0.052
	p value	**0.007**	0.745	0.457	0.636	**<0.001**	0.879

Table 6.4.3. Results of Mann-Whitney tests used to investigate differences between the degree of staining for iNOS and categorical clinical and cytological findings. Significant results ($p<0.05$) are highlighted in bold. w= Mann-Whitney statistic.

		Cough	Mucus	Clinical score	Neutrophilic Inflammation
% macrophages staining >0 for iNOS	w value	46.000	51.000	68.500	16.000
	p value	**<0.001**	0.287	0.322	0.579
% macrophages staining >1 for iNOS	w value	36.000	33.000	59.000	18.000
	p value	0.144	1.000	0.237	0.853
% macrophages staining >2 for iNOS	w value	25.000	27.000	63.000	65.000
	p value	0.256	0.400	0.738	0.250
% neutrophils staining >0 for iNOS	w value	49.000	35.000	67.000	21.000
	p value	0.523	0.6699	0.672	0.826
% neutrophils staining >1 for iNOS	w value	41.000	41.000	59.000	10.000
	p value	0.501	0.178	0.221	0.090
% neutrophils staining >2 for iNOS	w value	45.000	40.000	60.500	20.000
	p value	1.000	0.287	0.186	1.000
% lymphocytes staining >0 for iNOS	w value	44.500	33.000	66.500	0.120
	p value	0.935	1.000	0.830	96.800
%lymphocytes staining >1 for iNOS	w value	31.000	35.000	62.000	54.000
	p value	0.856	0.717	0.549	0.239
% lymphocytes staining >2 for iNOS	w value	45.000	34.000	60.500	18.000
	p value	1.000	0.803	0.186	1.000

6.5 Discussion

Correlation was observed between high percentages of neutrophils, macrophages and lymphocytes staining for iNOS and the percentage of eosinophils identified in BALF. However, in all but 1 case eosinophils were not identified and in that single case eosinophils only accounted for 3% of cells. This statistically significant finding is based upon very limited data therefore. In other species a number of associations between iNOS and eosinophil activity have been identified previously. Peroxynitrite increases airway hyper-responsiveness, respiratory epithelial damage and eosinophil activation in guinea pigs (Sadeghi-Hashjin *et al.* 1996). In models of allergic airway disease, iNOS knockout mice show reduced eosinophil tracking, plasma extravasation and airway obstruction (Xiong *et al.* 1999). Nitric oxide promotes production of chemokines for eosinophils in mice indicating the possibility of a positive-feedback loop with inflammatory cells producing NO that promotes further leukocyte recruitment (Ricciardolo *et al.* 2004). Immunohistochemistry has been used to identify 3-nitrotyrosine residues within tissue samples (Saleh *et al.* 1998) and biological fluids (Ohshima *et al.* 1999). 3-Nitrotyrosine is formed readily from hydrogen peroxide and nitrite in a process mediated by myeloperoxidase and eosinophil peroxidise (Kettle *et al.* 1997; Eiserich *et al.* 1998; Wu *et al.* 1999). 3-Nitrotyrosine contributes to cellular dysfunction and is a specific biomarker of nitrosative stress.

Other significant correlations were seen between iNOS staining in particular cell types and indicators of respiratory disease. Although plausible explanations for these relationships could be proposed there was no apparent pattern to their occurrence and they may have resulted by chance due to the large number of statistical tests being performed. Furthermore group sizes were frequently uneven and hence tests were often being made with only 2 or 3 horses in one category. For example, only 2 horses had normal neutrophil percentages in BALF and only 2 had positive (>0) clinical scores.

Inducible NOS has been identified in a diverse range of cell types and is induced by a range of pro-inflammatory mediators (Fischer *et al.* 2002) that would be present in the lower airway during RAO and IAD. In other species increases in iNOS expression results in attenuated airway hyperresponsiveness and reduced bronchoconstriction and a detectable increase in NO metabolites in BALF (Liu *et al.* 1993; Schuiling *et al.* 1998; Toward and Broadley 2000; Samb *et al.* 2001; Hjoberg *et al.* 2004). Furthermore NO signalling mediates airway secretion in response to inflammatory mediators such as histamine, TNF-α, methacholine and bradykinin (Adler *et al.* 1995). Production of NO is

increased in response to inflammatory cytokine production. In laboratory animals, macrophages have been shown to produce nitrite in a dose- and time- dependent manner following activation with lipopolysaccharide and IFN-γ, a process that is blocked by glucocorticoids (Jorens *et al.* 1991). Other cytokines, including TGF-β, IL-4 and IL-10 inhibit the expression of iNOS (Nijkamp and Folkerts 1994). In addition, NO reduces the production the inflammatory cytokines TNF, IL-1, MIP-1a from human macrophages during inflammatory conditions, suggesting a modulatory role in disease (Thomassen *et al.* 1997). Nitric oxide also suppresses antigen-induced degranulation, mediator release and cytokine expression by mast cells. *In vivo*, NO inhibits mast cell-dependent histamine-induced vasodilation, vasopermeation and leukocyte-endothelial cell attachment (Coleman 2002).

In a previous report of iNOS expression in horses, those animals with SPARAO had a higher percentage of bronchial epithelial cells staining for iNOS than non-affected horses (Costa *et al.* 2001). Interestingly, staining of peribronchiolar leukocytes for iNOS was no greater for horses with SPARAO than for control horses. This is consistent with the results above in which no clear association could be determined between the degree of iNOS staining in leukocytes and clinical signs of disease. In the study by Costa *et al.* (2000), nitric oxide concentrations in plasma, BALF and ELF were also determined whilst iNOS, nitrotyrosine and nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) were localised in formalin-fixed lung specimens using immunohistochemical and histochemical staining. Plasma concentration of NO in affected horses was non-significantly increased over non-affected controls. Nitric oxide concentrations in BALF and ELF were not different between groups. Immunoreactivity of iNOS was increased in bronchial epithelial cells in 3 of 5 lung lobes. Staining for NT and NADPHd did not differ between the two groups (Costa *et al.* 2000b).

The absence of a detectable increase in NO production in the lower respiratory tract in the study by Costa *et al.* (2000) was unexpected given an increase in exhaled NO has been reported in multiple studies of human patients suffering from asthma (Alving *et al.* 1993; Kharitonov *et al.* 1994; Massaro *et al.* 1995; Kharitonov *et al.* 1996a).

Further investigation into the relationships between clinical respiratory disease, iNOS expression and activity and the products of iNOS expression are required. The findings of the current study are limited by the number of horses included and by the lack of samples from horses considered to be free from lower airway disease. Ethical constraints prohibited sampling from normal horses and few of the horses sampled turned out to be free from

respiratory disease. The majority of cases included in the current study were suffering from RAO. Ethical constraints also prevented collection of pulmonary or bronchial biopsies from horses in the current study as they are rarely justified clinically. Future studies ought to use more objective methods that can be demonstrated to be repeatable given the concerns over the repeatability of the methods used in this study. Furthermore, methods of assessing activity and expression, or at least greater than one means of assessing expression, should be incorporated. Following on from the work by Costa *et al.* (2000) it would have been useful to have compared immunostaining in samples of tissue and BALF collected simultaneously. Attempts were made to collect tissue samples from horses with RAO and horses with no evidence of respiratory disease however insufficient samples were collected.

The effects of timing on sample collection and immunostaining are unknown. As with HO-1 the effects of iNOS expression in different stages of respiratory inflammation could be investigated further however challenge studies in horses known to suffer from RAO would probably be required to ensure sample collection early in the course of the inflammatory response.

CHAPTER SEVEN

INTERRACTIONS BETWEEN iNOS AND HO-1 EXPRESSION IN THE INFLAMMATORY CELLS OF EQUINE BALF

7.1 Introduction

Haem oxygenase-1 and iNOS are up-regulated in similar disease states and often by the same trigger factors. It is has been suggested therefore that there are complex interactions between the 2 enzyme systems (Ryter and Otterbein 2004). Induction of HO-1 may inhibit production of NO primarily by reducing the concentration of available haem which is a constituent of iNOS (Maines 1997). There may also be competition between the 2 systems for NADPH and electrons (Maines 1997).

By contrast, NO and peroxynitrite have been demonstrated to up-regulate HO-1 expression (Foresti and Motterlini 1999; Foresti *et al.* 1999; Liang *et al.* 2000) and it has been suggested that CO may induce iNOS and assume many of the roles of NO under oxidative conditions (Foresti and Motterlini 1999; Zuckerbraun *et al.* 2003). In turn, iNOS induces HO-1 thus resulting in a positive feedback system (Zuckerbraun *et al.* 2003). The effects of CO may be tissue specific as CO has been associated with inhibition of NOS in other models of tissue injury (Nakao *et al.* 2003).

Nitric oxide has been shown both to inhibit (Willis 1995) and activate (Motterlini *et al.* 1996) HO activity. Nitric oxide has even been suggested to be the strongest inducer of HO-1 (Motterlini *et al.* 1996) and it is therefore possible that many of the effects of NO may be modulated via HO-1 (Ryter and Otterbein 2004). Inhibitory activity of NO on HO-1 has also been reported and is likely due to NO orchestrated oxidative damage to the proteins of haem-oxygenase and its substrate haem. Nitric oxide has a higher affinity for haem than oxygen has for haem and the presence of NO therefore prevents oxidation. Conversely, the presence of free radicals may induce HO-1 activity in other conditions.

Interactions between HO-1 and iNOS are therefore complex and at times contradictory. Relative levels of synergy and competition may be tissue specific and be influenced by the nature of the inflammatory response.

7.2 Aims

The aims of this study were to investigate whether a relationship exists between HO-1 and iNOS expression in leukocytes from equine BALF. It was hypothesised that a positive linear relationship would exist between the expression of HO-1 and iNOS.

7.3 Methods

Cases presented for the investigation of respiratory disease or poor performance were examined and clinical findings recorded as outlined in Chapter 2.2. Samples of BALF were collected and processed as described in Chapter 2.3. In brief, following sedation BALF was collected using a commercially available BAL tube. Phosphate buffered saline was instilled into the distal airways, aspirated via the tube and the retrieved fluid was pooled to give a single sample. Slides were prepared for immunostaining within 4 hours of collection. The slides were stained for HO-1 and iNOS using rabbit polyclonal antibodies utilizing a Dako EnVision kit K4011 (Dako, Ely, UK) as per manufacturer's instructions with all incubations at room temperature as outlined in Chapter 2. Stained BALF slides from 14 horses was examined and the degree of staining determined for a representative sample of cells as described in Chapter 2.6. Staining was graded on a 0-3 scale for each cell. In 3 horses results were not obtained because staining was not of sufficient quality for one of the stains and statistical analysis was therefore performed on the results from 11 horses.

Results of immunostaining for HO-1 and iNOS were compared using Pearson's correlation.

7.4 Results

The results of immunostaining for HO-1 and iNOS were tabulated (Table 7.4.1) and displayed graphically (Figures 7.4.1, 7.4.2 and 7.4.3). The results of Pearson's correlations for each cell type at each grade of staining are displayed in Table 7.4.2. Statistically

significant correlations were identified for all grades of staining in macrophages with r values of 0.633, 0.724 and 0.892 indicating a strong correlation. Statistically significant correlations were also identified in neutrophils staining >2 and lymphocytes staining >1 and >2. These correlations were also strong with r being above 0.6.

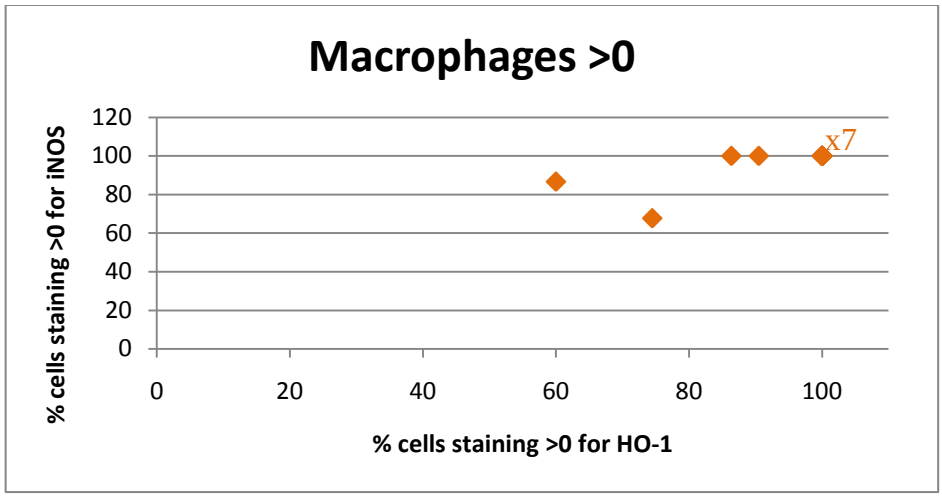
Table 7.4.1. The relationship between HO-1 and iNOS expression in leukocytes from equine BALF.

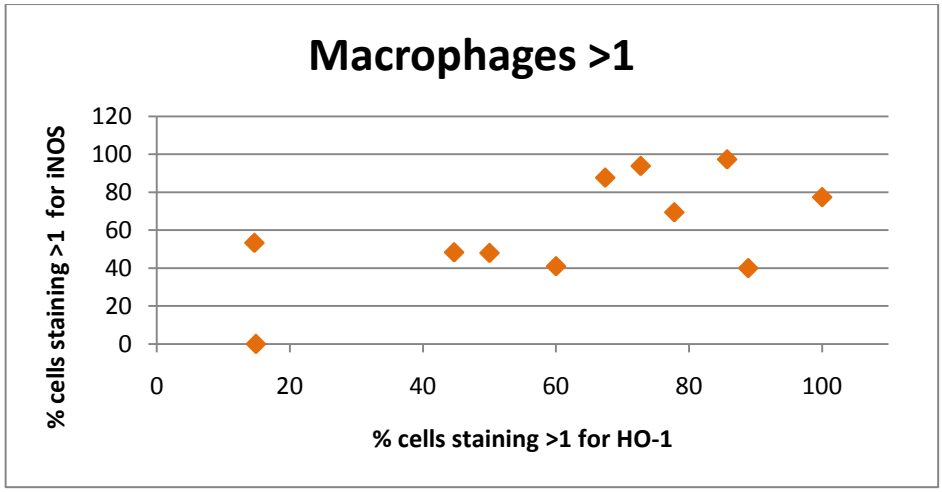
	Macrophages						Neutrophils							
	%>0		%>1		%>2		%>0		%>1		%>2		%>0	
Case	HO-1	iNOS	HO-1	iNOS	HO-1	iNOS	HO-1	iNOS	HO-1	iNOS	HO-1	iNOS	HO-1	iN
2	100.0	100.0	77.8	69.4	24.4	11.1	50.0	100.0	0.0	0.0	0.0	0.0	46.2	1
3	90.5	100.0	85.7	97.4	76.2	89.5	100.0	100.0	100.0	38.9	75.0	11.1	75.0	8
4	86.4	100.0	72.7	93.9	1.5	0.0	66.7	100.0	33.3	0.0	33.3	0.0	31.1	4
5	60.0	67.7	14.9	0.0	0.0	0.0	100.0	100.0	62.5	0.0	0.0	0.0	72.2	7
6	100.0	100.0	44.7	48.3	4.3	8.3	100.0	100.0	62.5	0.0	0.0	0.0	21.3	1
8	100.0	100.0	67.4	87.7	30.4	15.8	100.0	100.0	20.0	17.2	0.0	0.0	34.2	7
9	74.5	86.7	14.7	53.3	5.3	0.0	100.0	93.0	100.0	68.0	50.0	0.0	54.5	0
10	100.0	100.0	100.0	77.4	0.0	8.1	100.0	100.0	0.0	61.8	0.0	5.9	100.0	10
11	100.0	100.0	60.0	41.0	7.3	24.6	100.0	42.9	0.0	0.0	0.0	0.0	6.6	1
12	100.0	100.0	88.9	40.0	28.9	8.9	100.0	100.0	50.0	100.0	0.0	0.0	54.7	9
13	100.0	100.0	50.0	48.0	0.0	0.0	95.8	47.4	45.8	26.3	0.0	0.0	100.0	1

Table 7.4.2. The results of Pearson's correlations used to investigate relationships between the degree of staining for HO-1 and iNOS in leukocytes from equine BALF. Significant results are highlighted in bold.

	Macrophages		Neutrophils		Lymphocytes	
	r value	p value	r value	p value	r value	p value
% staining > 0	**0.724**	**0.012**	-0.188	0.579	0.373	0.259
% staining > 1	**0.633**	**0.036**	0.296	0.376	**0.642**	**0.033**
% staining > 2	**0.892**	**<0.001**	**0.615**	**0.044**	**0.778**	**0.005**

Figure 7.4.1 The relationship between HO-1 and iNOS staining in macrophages from equine BALF. Each scatter plot represents the degree of staining above a certain grade.





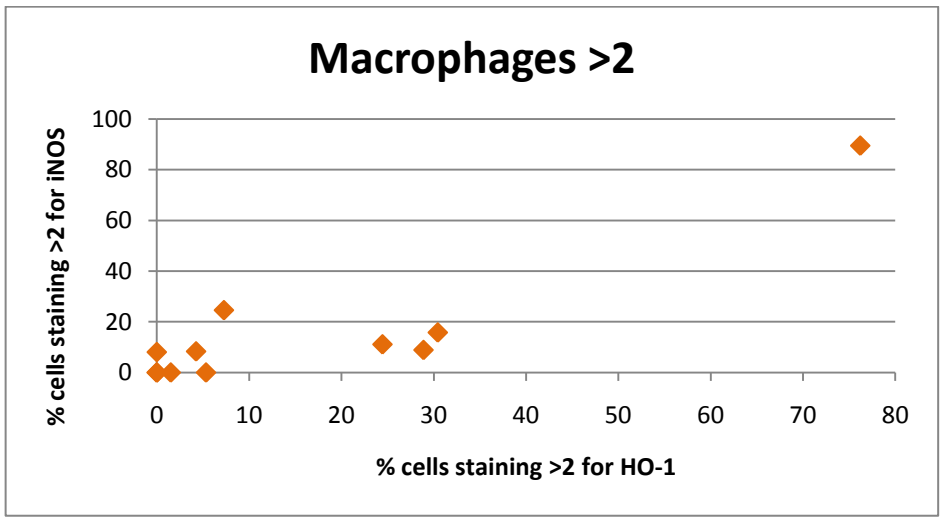
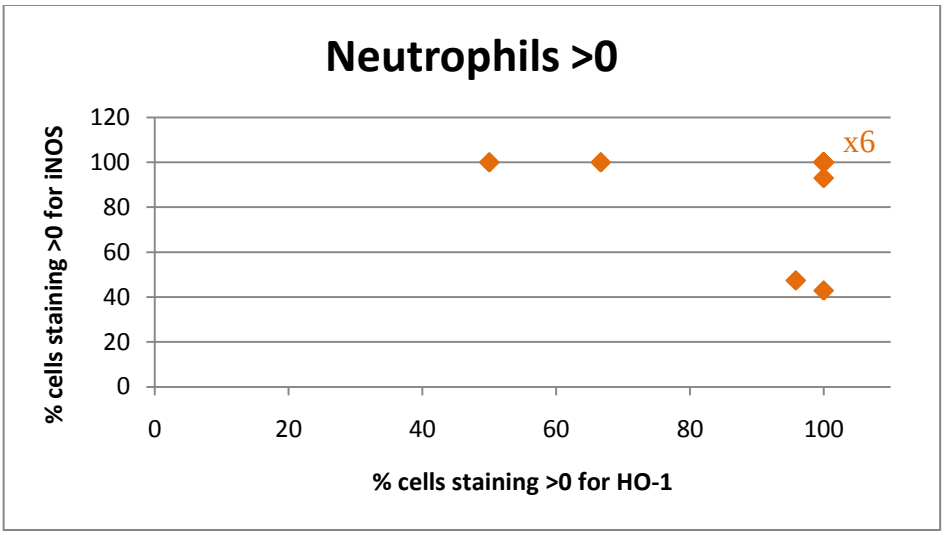
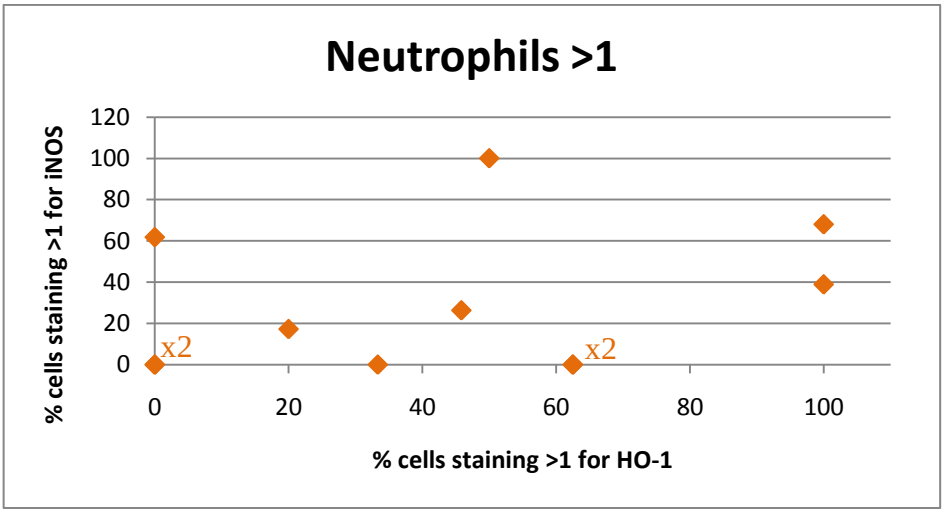


Figure 7.4.2. The relationship between HO-1 and iNOS staining in neutrophils from equine BALF. Each scatter plot represents the degree of staining above a certain grade.





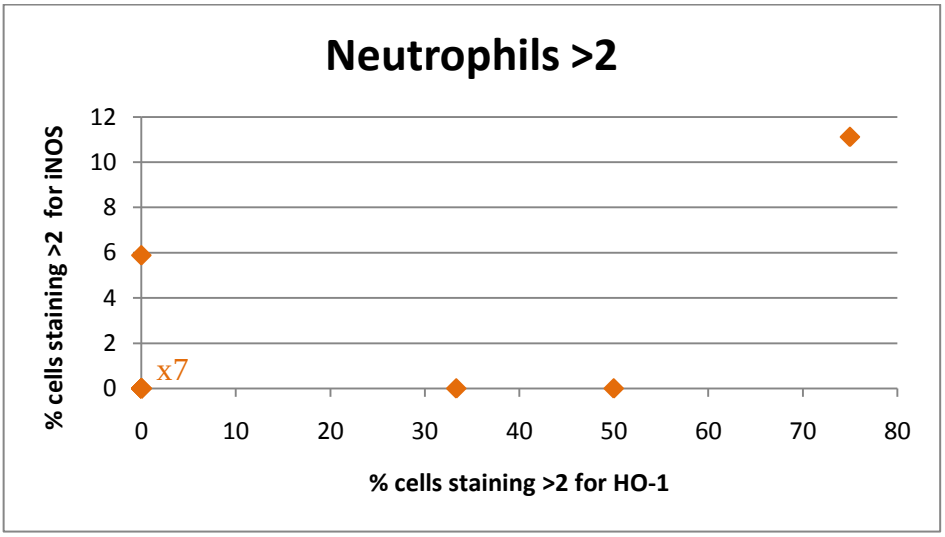
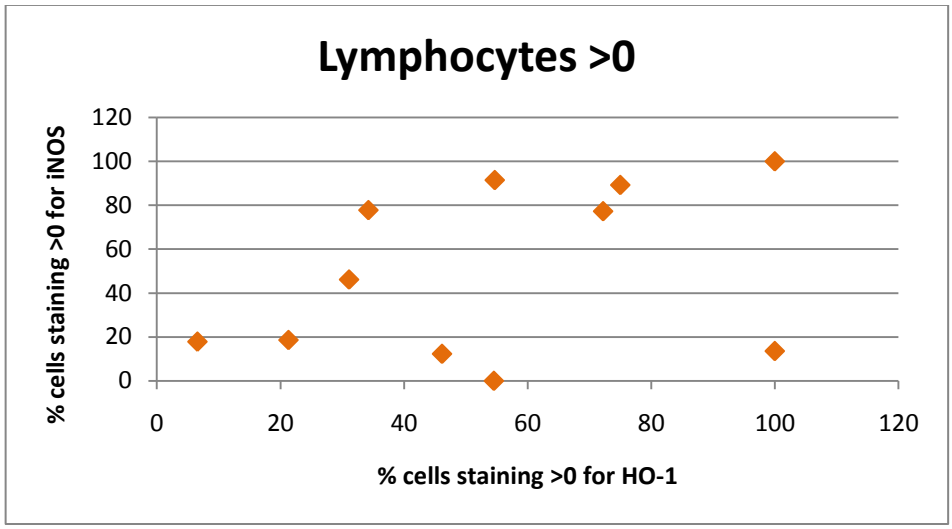
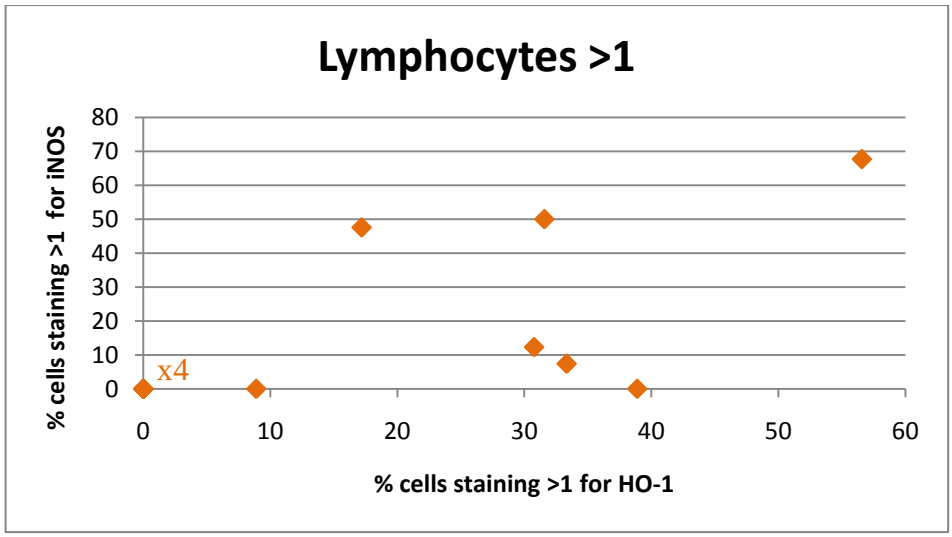
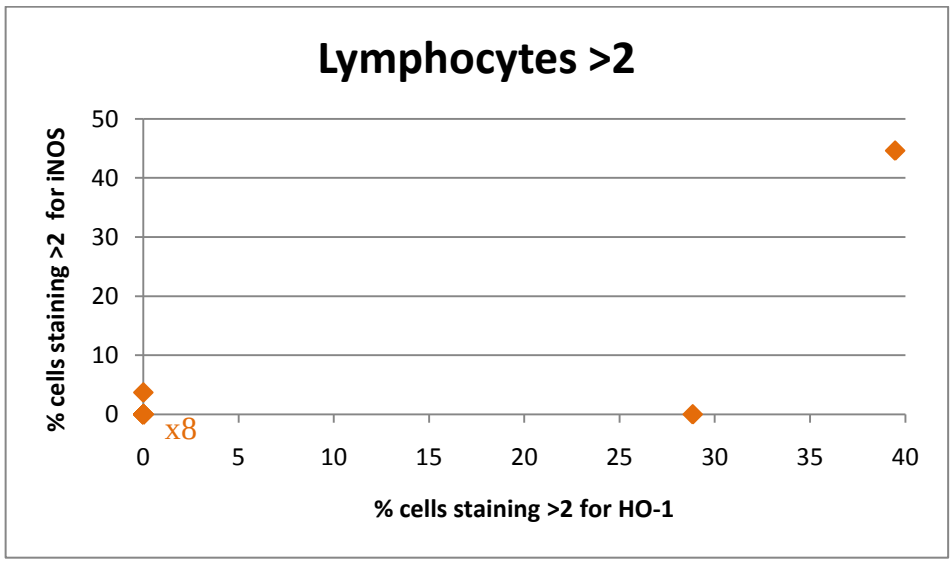


Figure 7.4.3. The relationship between HO-1 and iNOS staining in lymphocytes from equine BALF. Each scatter plot represents the degree of staining above a certain grade.







7.5 Discussion

In the current study there were statistically significant correlations between the degree of HO-1 and iNOS staining for 6 out of the 9 categories investigated. A positive correlation was identified for all degrees of macrophage staining and the 3 r values were between 0.6 and 0.9 indicating a high level of correlation. Significant correlations were also identified for the percentage of neutrophils staining > 2 and for the percentage of lymphocytes staining >1 and >2. The r values were greater than 0.6 indicating a strong correlation. It was a concern however that the data set was small and the presence of one or two outlying results may have had a large influence on the results of statistical analysis in some of the categories. Further work with greater case numbers should be performed.

To the author's knowledge, associations between expression of HO-1 and iNOS in leukocytes in equine BALF have not been reported previously. Correlation between the two systems was not unexpected as significant and complex interactions between the two systems are known to exist in other species (Ryter and Otterbein 2004). The findings of the current study may indicate that expression of HO-1 and iNOS in the lower airways of horses are induced by the same or similar initiating factors. This was expected as many of the initiating factors that have been identified are common to both enzyme systems and would be present as a consequence of inflammatory disease of the lower airways. Inducible NOS may be induced by endogenous factors including the pro-inflammatory cytokines TNFα, IFN-γ and interleukin1-β (IL-1 β) or exogenous factors such as bacteria, bacterial toxins (especially lipopolysaccharide), viruses, allergens and environmental pollutants (Morris and Billiar 1994; Yan *et al.* 1995; Yeadon and Price 1995; Goldman *et al.* 1996). In addition, hypoxia, oxidative injury and tumour cells may stimulate iNOS expression (Yan *et al.* 1995; Yeadon and Price 1995; Goldman *et al.* 1996). Haem oxygenase-1 gene expression is induced via a diverse range of signalling pathways (Maines 1997) and notably is induced strongly by agents and conditions associated with oxidative stress. In addition, a number of important redox-independent pathways are also involved in up-regulating HO-1 gene expression.

Inflammation, infection, sepsis and lipopolysaccharide all result in ROS production. *In vitro*, the pro-inflammatory cytokines IL-1, IL-6 and TNF-α induce HO-1 expression (Rizzardini *et al.* 1993; Terry *et al.* 1999). In addition, growth factors (including transforming growth factor-β and platelet derived growth factor) induce HO-1 expression in association with the generation of ROS (Kutty *et al.* 1994; Durante *et al.* 1999). In addition to cytokine and growth factor influences on HO-1 expression, hypoxia alters the

iron and intracellular redox equilibrium activating HO-1 gene expression (Motterlini *et al.* 2000). Conversely, hyperoxia is associated with increased production of mitochondrial ROS and oxidative lung injury (Freeman and Crapo 1981).

Alternatively induction of one of the proteins may induce the other. Nitric oxide, the product of iNOS has been demonstrated to up-regulate HO-1 expression (Foresti and Motterlini 1999; Foresti *et al.* 1999; Liang *et al.* 2000) and it has been suggested that CO may assume many of the roles of NO under oxidative conditions (Foresti and Motterlini 1999). Motterlini *et al.* (1996) suggested that NO might be the strongest inducer of HO-1 and other authors have proposed that many of the effects of NO may be modulated via HO-1 (Ryter and Otterbein 2004). In addition, CO, one of the principal products of HO-1, has been shown to induce iNOS in hepatocytes which in turn induces HO-1 (Zuckerbraun *et al.* 2003). However, changes in NO expression did not appear to modulate HO-1 expression in one human study (Brunetti *et al.* 2006).

A stronger association between iNOS and HO-1 expression in macrophages than other cell types was found. Macrophages are the most consistent source of HO-1 and iNOS in previous studies of human and laboratory animal respiratory physiology and disease (Kitada *et al.* 2001; Pechkovsky *et al.* 2002; Brunetti *et al.* 2006). Absence of an association in some cases and in other cell types might be due to the effects of sample timing relative to the stage of the disease process. Willis *et al.* (1996) speculated that during tissue inflammation there may be sequential induction of enzyme systems with induction of iNOS being followed by increased expression of enzymes with anti-inflammatory effects, including HO-1. Furthermore whilst HO-1 may be identified in healthy tissue from equine lungs (Wyse *et al.* 2006) under healthy conditions iNOS expression may not be detected (Buttery *et al.* 1994).

Previous studies in other species provide evidence of mutual inhibition as well as stimulation (Willis 1995; Wang *et al.* 2003). Further investigations of the interactions between the two enzyme systems are indicated in order to investigate relationships in different disease states and at different stages of disease. Future studies ought to use methods with a more established means of objectively assessing expression, and preferably activity, given the concerns over the repeatability of the methods used in this study. A greater number of horses should be used than were used in the current study with a more comprehensive and representative sample of animals suffering from different disease states and those considered free of respiratory disease. Future studies also ought to investigate

levels of activity for both enzyme systems rather than merely looking at expression. Expression may not provide a reliable indication of enzyme activity.

CHAPTER EIGHT

CORRELATION BETWEEN MARKERS OF OXIDATIVE STRESS ASSESSED BY IMMUNOSTAINING AND EXHALED BREATH CONDENSATE ANALYSIS

8.1 Introduction

Biomarkers in exhaled breath condensate (EBC) have been investigated widely in studies of human respiratory physiology and diseases, most notably asthma (Jobsis *et al.* 1997; Ho *et al.* 1999; Hanazawa *et al.* 2000; Niimi *et al.* 2004 Brunetti *et al.* 2006). There are many constituents of exhaled breath condensate that may be influenced by lower airway inflammation including H_2O_2, NO, adenosine, arachidonic acid metabolites, cytokines, leukotrienes, 8-isoprostane, pH and ammonia (Horvath 2005). Hydrogen peroxide and pH are the most commonly measured in humans (Horvath *et al.* 2005). Hydrogen peroxide is produced primarily by activated inflammatory cells in the airways as a result of oxidative stress (Babior 2000). Hydrogen peroxide concentration in EBC has been shown to correlate with presence and severity of asthma (Antczak *et al.* 2000; Loukides *et al.* 2002) and pH has been demonstrated to be a reliable indicator of inflammatory respiratory disease in man (Kostikas *et al.* 2002; Vaughan *et al.* 2003b; Borrill *et al.* 2005).

In one study performed in horses, H_2O_2 concentration in EBC was correlated with ascorbic acid concentration in PELF (Deaton *et al.* 2004b). However, further studies which compared horses with airway inflammation to normal controls failed to find significant differences in exhaled hydrogen peroxide concentrations (Deaton *et al.* 2005a; Wyse *et al.* 2005, Duz *et al*. 2009). Prolonged exposure to inflammatory stimuli may be required to deplete antioxidant capacity (Deaton 2006).

Haem oxygenase-1 is the major stress protein induced by H_2O_2, (Keyse and Tyrrell 1989). In addition, any agents that directly or indirectly generate ROS or alter the redox equilibrium will induce HO-1 expression; HO-1 is therefore a general marker of oxidative

stress (Applegate *et al.* 1991). Hypoxia, oxidative injury and tumour cells may stimulate iNOS expression (Goldman *et al.* 1996; Yan *et al.* 1995; Yeadon and Price 1995).

8.2 Aims

To investigate the relationship between HO-1 and iNOS staining in leukocytes from equine BALF and markers of oxidative stress in exhaled breath condensate. It was hypothesised that expression of HO-1 and iNOS would be higher in horses which had greater concentrations of H_2O_2 and lower pH in EBC ie a positive correlation would be observed with H_2O_2 and a negative correlation with pH.

8.3 Methods

Horses presented for the investigation of respiratory disease or poor performance were examined and clinical findings recorded as outlined in Chapter 2.2. Samples of BALF were collected and processed as described in Chapter 2.3. In brief, following sedation BALF was collected using a commercially available BAL tube. Phosphate buffered saline was instilled into the distal airways, withdrawn via the tube and the fluid obtained pooled to give a single sample. Slides were prepared for immunostaining within 4 hours of collection. The slides were stained for HO-1 using rabbit polyclonal antibodies utilizing a Dako EnVision kit K4011 (Dako, Ely, UK) as per manufacturer's instructions with all incubations at room temperature as outlined in Chapter 2. Stained BALF was examined and the degree of staining determined for a representative sample of cells as described in Chapter 2.6. Staining was graded on a 0-3 scale for each cell. From these results the following calculations were made:

i) the percentage of cells with any stain uptake (i.e. grades 1-3),

ii) the percentage of cells staining greater than 1 (i.e. grades 2 and 3),

iii) the percentage of cells staining greater than 2 (i.e. grade 3).

Exhaled breath condensate was collected according to a method that has been published previously (Duz *et al.* 2009). Collection was performed before horses were sedated for the BAL procedure. Eight of the horses used for the analysis of BALF were available for collection of EBC. For one of the 8, iNOS staining was unsatisfactory and comparisons between EBC and iNOS expression were therefore performed for 7 cases and comparisons between EBC and HO-1 performed for 8 cases. A face mask was connected via tubing to a polypropylene condensation chamber immersed in ethanol/liquid nitrogen slurry to

maintain a temperature below -80°C. The face mask was constructed from a muzzle modified with fibreglass and incorporating two one-way non-rebreathing valves. A rubber diaphragm around the mask created an airtight seal with the horse's head. The non re-breathing valves were connected by a rubber flexible tube (Cranlea, UK) to the condensation chamber. Exhaled breath condensed on the internal surface of the polypropylene container. A one-way valve on the outlet of the condensation chamber prevented contamination of the sample with environmental air. Breath condensate was collected over a 10 minute time period. Collected samples of EBC were maintained on ice during transit to the laboratory.

Exhaled breath condensate was thawed and analysed immediately. Measurement of H_2O_2 concentration was performed using a spectrophotometric assay based on the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of horseradish peroxidise (HRP; Sigma-Aldrich, UK) modifying a published method (Gallati and Pracht 1985). In summary, 15 µl of HRP (75IU/ml), 140 µl of TMB in 0.42M citrate buffer (pH 3.8) and 140 µl of EBC were mixed at room temperature. The reaction was stopped after 10 minutes by addition of 15µl of 9M sulphuric acid. The product of the reaction was analysed immediately with an automated microplate reader (FLUOstar OPTIMA, BMG labtech, Aylesbury, UK) using a wavelength for absorbance of 450 nm. Exhaled breath condensate H_2O_2 concentration was calculated from a standard curve of known concentrations of H_2O_2 (from 0.1 to 10 µM) prepared fresh for each assay. The lower detection limit of the assay was 0.1 µM. Linearity of the standard curve was acceptable (r^2 > 99%) between 0 and 10 µM. The measurement was repeated 4 times for each sample and the final H_2O_2 concentration was calculated as the mean of the 4 measurements.

Measurement of pH of EBC samples was performed using a pH meter (pH21, Hanna Instruments Ltd) connected to a glass-body pH electrode with an accuracy of ± 0.02 pH (HI-2031B, Hanna Instruments Ltd).

The relationship between degrees of staining and the results of EBC analysis were investigated using Pearson's correlation.

8.4 Results

Collection of EBC and BALF was performed in 8 horses; 4 thoroughbreds and 4 other breeds. Ages ranged from 11 to 21 years, there were 5 mares and 3 geldings. Six of the 8

were diagnosed with chronic RAO and the remaining two horses were considered to be free from respiratory disease (horses 6 and 11). None of the 8 horses had received treatment prior to sampling. The results obtained are presented in Table 8.4.1 and the results of statistical analyses for HO-1 and iNOS staining are presented in tables 8.4.2 and 8.4.3 respectively.

Table 8.4.1 Results of HO-1 immunostaining in leukocytes from BALF and concentration of H_2O_2 and pH of EBC.

	EBC		HO-1 Staining								
Case	H_2O_2 (mMol)	pH	Macrophages			Neutrophils			Lymphocytes		
			%>0	%>1	%>2	%>0	%>1	%>2	%>0	%>1	%>2
2	9.7	5.83	100.0	77.8	24.4	50.0	0.0	0.0	46.2	30.8	28.8
3	6.3	4.85	90.5	85.7	76.2	100.0	100.0	75.0	75.0	56.6	39.5
6	3.3	6.1	100.0	44.7	4.3	100.0	62.5	0.0	21.3	0.0	0.0
7	2.4	4.84	100.0	100.0	57.7	100.0	100.0	33.3	0.0	0.0	0.0
9	2.6	4.41	60.0	14.7	5.3	100.0	100.0	50.0	54.5	0.0	0.0
11	5.0	7.74	100.0	60.0	7.3	100.0	0.0	0.0	6.6	0.0	0.0
12	2.4	5.64	100.0	88.9	28.9	100.0	50.0	0.0	54.7	17.2	0.0
13	0.9	5.53	100.0	50.0	0.0	95.8	45.8	0.0	100.0	33.3	0.0

Table 8.4.2 Results of iNOS immunostaining in leukocytes from BALF and concentration of H_2O_2 and pH of EBC.

	EBC		HO-1 Staining								
Case	H_2O_2 (mMol)	pH	Macrophages			Neutrophils			Lymphocytes		
			%>0	%>1	%>2	%>0	%>1	%>2	%>0	%>1	%>2
2	9.7	5.83	100.0	69.4	11.1	100.0	0.0	0.0	12.3	12.3	0.0
3	6.3	4.85	100.0	97.4	89.5	100.0	38.9	11.0	89.2	67.7	44.6
6	3.3	6.1	100.0	48.3	8.3	100.0	0.0	0.0	18.6	0.0	0.0
9	2.6	4.41	86.7	53.3	0.0	93.0	68.0	0.0	0.0	0.0	0.0
11	5.0	7.74	100.0	41.0	24.6	42.9	0.0	0.0	17.9	0.0	0.0
12	2.4	5.64	100.0	40.0	8.9	100.0	100.0	0.0	91.5	47.6	0.0
13	0.9	5.53	100.0	48.0	0.0	47.4	26.3	0.0	13.6	7.4	3.7

Table 8.4.3. The results of Pearson's correlations used to investigate relationships between HO-1 immunostaining in leukocytes from BALF and markers of oxidative stress in exhaled breath condensate. Statistically significant results are highlighted in bold.

		H_2O_2		pH	
		r value	p value	r value	p value
Macrophages	% staining > 0	0.135	0.750	0.448	0.265
	% staining > 1	0.273	0.513	0.009	0.984
	% staining > 2	0.287	0.491	-0.429	0.289
Neutrophils	% staining > 0	**-0.775**	**0.024**	-0.081	0.849
	% staining > 1	-0.444	0.270	**-0.821**	**0.012**
	% staining > 2	0.066	0.877	-0.675	0.066
Lymphocytes	% staining > 0	-0.106	0.803	-0.393	0.335
	% staining > 1	0.392	0.337	-0.256	0.540
	% staining > 2	**0.789**	**0.020**	-0.208	0.620

Table 8.4.4. The results of Pearson's correlations used to investigate relationships between iNOS immunostaining in leukocytes from BALF and markers of oxidative stress in exhaled breath condensate.

		H_2O_2		pH	
		r value	p value	r value	p value
Macrophages	% staining > 0	0.254	0.582	0.548	0.203
	% staining > 1	0.594	0.160	-0.473	0.283
	% staining > 2	0.407	0.364	-0.128	0.785
Neutrophils	% staining > 0	0.331	0.468	-0.570	0.181
	% staining > 1	-0.462	0.297	-0.557	0.194
	% staining > 2	0.297	0.518	-0.365	0.421
Lymphocytes	% staining > 0	0.024	0.960	-0.184	0.693
	% staining > 1	0.176	0.705	-0.369	0.416
	% staining > 2	0.547	0.160	-0.376	0.406

8.5 Discussion

Three statistically significant results were identified between expression of HO-1 in leukocytes from BALF and markers of inflammation in EBC. No significant correlations were identifieded between iNOS expression and EBC markers. A negative correlation was identified between neutrophils staining greater than 0 and H_2O_2 concentration in EBC whilst a positive correlation was identified for lymphocytes staining greater than 2 for HO-1 and H_2O_2 concentration in EBC. A negative correlation was identified between neutrophils staining greater than 1 and EBC pH. The positive correlation with H_2O_2 and negative correlation with pH were expected, however the negative correlation with H_2O_2 was unexpected and contradicted the other significant result for H_2O_2. Considering only 3 of the 36 correlations investigated were significant and the results were contradictory it is

unlikely that these results indicate a genuine association between HO-1 expression and markers in EBC.

There may be a number of explanations for these findings. First, the methods used to collect and analyse EBC may not be reliable. Second, the methods used to investigate expression of HO-1 and iNOS may not be reliable and third there may be no correlation between the expression of HO-1/iNOS and the constituents of EBC. It is likely that there are numerous biological processes that affect the composition of EBC. The methods under investigation in this study may be of minor importance.

In the present study the concentrations of H_2O_2 were much higher (by a factor of 10) than in previous studies (Deaton *et al.* 2004b; Wyse *et al.* 2005). This led to concern that the results may not be reliable. However, the methods used were different between studies and the methodology used in the current study was considered accurate when testing known concentrations of test substance and the inter-assay coefficient of variation was <0.05 consistent with good repeatability (Duz *et al.* 2009). Although comparison with previous studies was complicated by the disparity of the results we were confident that our results were accurate and repeatable. Inter- and intra-day variability in H_2O_2 concentration was high in horses with lower airway inflammation (data not shown) which may have been related to varied inflammatory responses to environmental challenges. The inter- and intra-day variability of HO-1 and iNOS expression could not be investigated for practical and ethical issues of repeated collection of BALF from horses and this may have led to a disparity of the results. As this is the first study to investigate HO-1 and iNOS expression in samples from the equine respiratory tract the variation in expression over time is unknown. Exhaled breath condensate was collected within two hours of BALF collection to minimize these potential effects.

The importance of analysis of EBC as a tool in the investigation of equine respiratory disease remains to be determined. The analysis of EBC has not been used widely for the investigation of respiratory physiology or inflammatory diseases of the lower respiratory tract in horses. In the current study and previous investigations, significant differences between animals with clinical disease and controls have not been evident (Deaton *et al.* 2005b; Wyse *et al.* 2005; Duz *et al.* 2009). However, Duz *et al.* (2009) did find a trend for decreased pH of EBC in horses with lower airway inflammation. It is yet to be determined whether analysis of EBC is a reliable means of assessing respiratory tract inflammation and perhaps correlation with other markers of inflammation and oxidative injury such as HO-1 and iNOS expression should not be expected. Previous human studies have failed to

demonstrate associations between levels of HO-1 and levels of its exhaled products such as CO (Brunetti *et al.* 2006).

Although H_2O_2, HO-1 and iNOS are all associated with conditions of oxidative stress numerous other substances and processes may induce HO-1 and iNOS expression (Babior 2000; Otterbein and Choi 2000; Horvath *et al.* 2001). It is possible that in the inflamed equine lower airway, oxidative processes are of relatively minor importance in the induction of HO-1 and iNOS and hence there is a disparity between these different markers of oxidative injury. It is notable that in EBC there was no significant association between H_2O_2 and pH both of which ought to be markers of oxidative stress (Duz *et al.* 2009). Numerous compounds are present within EBC such as nitric oxide, adenosine, arachidonic acid metabolites, cytokines, 8-isoprostane and ammonia (Horvath *et al.* 2005). Although H_2O_2 is the compound most frequently measured in human studies of EBC (Loukides *et al.* 2002; Horvath *et al.* 2005) it may be too simplistic to investigate this single marker of oxidative stress within a "soup" of other potential markers. In human airways, H_2O_2 is produced by epithelial cells of the conducting airways (Schleiss *et al.* 2000) in addition to inflammatory cells which may be influential as iNOS and HO-1 expression were only determined in cells collected from the distal airways.

A limitation of the current study was the absence of repeated sampling from the same horse. The stage in the disease process at which samples were collected may have influenced the results. It is possible that if analyses were performed at different stages in the disease process or even summated over a time period associations with constituents of EBC might have been identified. A further limitation of the current study was the assumption that expression of HO-1 or iNOS was a reliable means of assessing activity. The concerns previously expressed over the repeatability of the methods used for analysis also extend to the results in this chapter. Future studies ought to investigate activity in addition to expression and be performed with greater numbers of horses in order to substantiate our findings. The majority of horses in the current study were suffering from RAO. Future studies ought to contain more horses with other respiratory diseases and also a larger number of horses considered free of respiratory disease.

The effects of treatment for respiratory disease on HO-1 and iNOS expression should also be considered. None of the horses in the current study were treated with corticosteroids prior to sampling. Future studies could investigate the effects of corticosteroids on iNOS, HO-1 and EBC constituents. A glucocorticoid response element is present in the HO-1 promoter, and gene expression of HO-1 is inhibited by corticosteroids *in vitro* (Cantoni *et*

al. 1991; Lavrovsky *et al.* 1996). The reduction in iNOS expression known to result from corticosteroid therapy (Saleh *et al.* 1998) would be expected to result in reduced expression of HO-1.

In conclusion, neither the pH nor the H_2O_2 concentration of EBC appeared to be influenced by the degree of expression of HO-1 or iNOS in leukocytes from BALF. This may have been due to the sampling and analytical methods used and future studies using more precise methods should be performed.

CHAPTER NINE

GENERAL DISCUSSION, CONCLUSIONS AND SUGGESTIONS FOR FURTHER INVESTIGATION

9.1 Characterisation of HO-1 and iNOS in the Equine Respiratory Tract in Health and Disease

The results of the current study indicate that HO-1 and iNOS are expressed in leukocytes present in equine BALF. Further, methods that have not been used previously in the examination of equine BALF have been documented.

The first of investigation aimed to determine whether frozen BALF could be used for immunostaining. A significant correlation was determined in only 1 or the 6 tests of correlation performed; cells staining grade 3 for iNOS. For this type and degree of staining the correlation was not only significant but was linear with the r value being 0.988. However, this result was strongly influenced by the presence of a single strong positive result (case 3) and a large number of negative results. Overall, correlation was poor and as the preservation of the fresh samples was visibly superior it was assumed that error was introduced by freezing and thawing. When fresh and frozen cells were compared the frozen cells were smaller and irregular in appearance. Cells were commonly aggregated and distinction of individual cells proved difficult. The subjective assessment of staining in individual frozen cells was therefore less precise and background staining was also greater in the slides of frozen BALF. We suspected that constituents of the cytoplasm were contributing to the background staining and the freezing and thawing processes are suspected to have damaged the phospholipid cell membrane. Frozen BALF would appear to be unsuitable for immunocytochemistry and the results from cases in which only frozen data was available were omitted from further analysis For immunocytochemical staining of cells in BALF, processing of samples should be performed shortly after collection of BALF without freezing.

In order to ensure the results of subsequent investigations would be accurate the repeatability of our methods was investigated and intra-observer variability analysed.

Unfortunately, definitive conclusions over the repeatability of these methods could not be reached and this may have influenced the validity of conclusions drawn in later chapters. Furthermore, our failure to investigate activity of HO-1 and iNOS, concusions were based upon a single assessment of expression in each sample. The standard deviation from the mean was greater overall for neutrophils than for other cell types. The reason why neutrophil counts were more variable is uncertain. There did not appear to be any differences in repeatability between staining for iNOS and HO-1. We could be confident therefore that any variation noted in subsequent chapters was likely to be a true finding and not due to errors introduced during sample processing and analysis.

The degree of HO-1 staining in different types of leukocytes from BALF was then compared to known markers of airway inflammation. The percentage of neutrophils staining greater than 1 and greater than 2 for HO-1 correlated with both respiratory rate and clinical score but not with other markers of inflammation such as neutrophil percentage. When assessing equine respiratory disease the number of neutrophils as a percentage of the total number of leukocytes in BALF is considered a reliable means of assessing the severity and correlates with histopathological changes in RAO (Larson and Busch 1985). It was therefore surprising that whilst HO-1 expression appeared to be related to clinical indicators of respiratory disease there was no relationship with relative neutrophilia. The significant associations that were identified between the percentage of neutrophils staining greater than 1 and 2 and clinical score are of questionable clinical relevance as only 3 horses actually had an abnormal clinical score and hence analysis was based upon very small numbers. Further investigations with more horses demonstrating more marked evidence of respiratory dysfunction at rest ought to be performed. No statistically significant associations were identified between HO-1 expression and the presence of a cough or tracheal mucus. Other associations between immunostaining of specific leukocyte types in BALF and clinical findings of respiratory disease were found also however they were of questionable relevance as eosinophils were only identified in 2 horses and results were therefore based upon very limited data.

Correlation was observed between high percentages of neutrophils, macrophages and lymphocytes staining for iNOS and the percentage of eosinophils identified in BALF. In other species a number of associations between iNOS and eosinophil activity have been identified previously; however, in our study this statistically significant result was based uponthe presence of eosinophils in only one horse. Other significant correlations were seen between iNOS staining in particular cell types and indicators of respiratory disease. Although plausible explanations for these relationships could be proposed there was no

apparent pattern to their occurrence and they may have resulted by chance due to the large number of statistical tests being performed. Furthermore group sizes were frequently uneven and hence tests were often being made with only 2 or 3 horses in one category. For example, only 2 horses had normal neutrophil percentages in BALF and only 2 had positive (>0) clinical scores.

In the current study there were statistically significant correlations between the degree of HO-1 and iNOS staining for 6 out of the 9 categories investigated. A positive correlation was identified for all degrees of macrophage staining and the 3 r values were between 0.6 and 0.9 indicating a high level of correlation. Significant correlations were also identified for the percentage of neutrophils staining > 2 and for the percentage of lymphocytes staining >1 and >2. The r values were greater than 0.6 indicating a strong correlation. It was a concern however that the data set was small and the presence of one or two outlying results may have had a large influence on the results of statistical analysis in some of the categories. Further work with greater case numbers should be performed. To the author's knowledge, associations between expression of HO-1 and iNOS in leukocytes in equine BALF have not been reported previously. Correlation between the two systems was not unexpected as significant and complex interactions between the two systems are known to exist in other species (Ryter and Otterbein 2004).

Expression of HO-1 and iNOS in leukocytes from BALF was compared to markers of oxidative stress in EBC. Three statistically significant results were identified between expression of HO-1 but no significant results were identifieded between iNOS expression and EBC markers. A negative correlation was identified between neutrophils staining greater than 0 and H_2O_2 concentration in EBC whilst a positive correlation was identified for lymphocytes staining greater than 2 for HO-1 and H_2O_2 concentration in EBC. A negative correlation was identified between neutrophils staining greater than 1 and EBC pH. The positive correlation with H_2O_2 and negative correlation with pH were expected, however the negative correlation with H_2O_2 was unexpected and contradicted the other significant result for H_2O_2. Considering only 3 of the 36 correlations investigated were significant and the results were contradictory it is unlikely that these results indicate a genuine association between HO-1 expression and markers in EBC.

The evidence of expression of HO-1 in leukocytes from the lower airways complements the findings of Wyse *et al.* (2006) who identified expression of HO-1 in type II alveolar cells, macrophages and neutrophils in sections of lung harvested from healthy horses. Several studies have investigated the expression of HO-1 in the lungs of humans. In bronchial biopsies from human subjects there was no difference in expression of HO-1 between normal and asthmatic patients and between asthmatic patients treated with corticosteroids and those that were untreated (Brunetti *et al.* 2006). By contrast, levels of HO-1 expression in macrophages from sputum have been demonstrated to be increased in asthmatic patients compared to healthy controls (Horvath *et al.* 1998). Furthermore, in a study comparing smoking and non-smoking human subjects, immunostaining was identified predominantly in alveolar macrophages (a median of 36% staining positive in smokers and 13% in non-smoking subjects) (Maestrelli *et al.* 2001). The majority of positive cells were present within the alveolar spaces rather than the bronchial epithelium; positive cells being mostly macrophages (Maestrelli *et al.* 2001).

In human airways HO-1 is distributed widely in the airway sub-mucosa, particularly within macrophages (Brunetti *et al.* 2006). Immunoreactivity of positive cells in that study was intense and non-granular yet no particular association with the cell membrane or specific organelle was observed (Brunetti *et al.* 2006). In another study, HO-1 expression was not identified in subepithelial T lymphocytes (Horvath *et al.* 1998). In mice, HO-1 immunostaining of macrophages, eosinophils, neutrophils, CD4+ and CD8+ T cells was determined by double immunostaining and confocal fluorescence (Kitada *et al.* 2001). The majority of HO-1 positive cells in tissue sections and BALF were macrophages (Kitada *et al.* 2001). Staining was diffuse but centered on the Golgi apparatus and perinuclear region.

Expression of iNOS has been investigated in both equine health and disease. Horses with SPARAO had a higher percentage of bronchial epithelial cells staining for iNOS than disease-free horses in one study (Costa *et al.* 2001). In that study, staining of peribronchiolar leukocytes for iNOS was no greater for horses with SPARAO than for control horses. This is consistent with the findings of our study in which no clear association could be determined between the degree of iNOS (or HO-1) staining in leukocytes and clinical signs of disease. These findings may indicate that leukocytes are not the predominant source of iNOS and HO-1 in the equine respiratory tract. In asthmatic humans and animal models of airway hyperresponsiveness, iNOS is believed to be synthesized principally by bronchial epithelial cells and macrophages (Barnes and Liew 1995). In the present study, macrophages stained more frequently for iNOS than did other

leukocytes which suggests that macrophages may be a significant source of iNOS in horses as they are in man.

9.2 Study Limitations and Suggestions for Further Investigations

9.2.1 Use of Frozen Samples

The number of horses recruited to the current study was limited by the discovery that slides prepared from frozen samples of BALF were unsuitable for immunocytochemistry. It was unfortunate that this was not determined prior to sample collection, however, where possible samples fresh samples were collected from the outset. Frozen samples were collected for logistical reasons because cytospin preparations could only be produced at the University of Glasgow. Samples collected at The Liphook Equine Hospital and samples collected at the University of Glasgow when the author was not available to process them were snap frozen in liquid nitrogen and maintained at -80°C for subsequent processing. It was assumed that cell preservation would have been adequate for immunocytochemistry as in a previous study, cytospun BALF slides were frozen and later thawed for immunostaining (Horvath *et al.* 1998). The fixing of cells during cytospin preparation may mitigate the deleterious effects of freezing resulting in an acceptable quality of immunostaining in the study by Horvath *et al.* (1998) and not in the current study.

The frozen cells were smaller and irregular in appearance when compared to fresh samples and cells were commonly aggregated making identification of individual cells difficult. Staining of background material was also greater in the slides of frozen BALF and it was presumed that constituents of the cytoplasm of damaged cells were contributing to the background staining. The freezing and thawing processes were presumed to have resulted in damage to the cell membrane and loss of cytoplasm into BALF. It is possible that when freezing is performed on cells that are already fixed in a cytospin preparation, loss of cytoplasmic contents is reduced or prevented and cytoplasmic granules containing HO-1 and iNOS remain within the limits of the cell despite damage to its membranes.

If further work is performed in this area cryopreservation techniques should be utilised. Foetal bovine serum may be added to 10% DMSO to enhance the survival of some cell lines and ought to be effective in preserving equine leukocytes. BALF could be centrifuged and the cell pellet resuspened in a mixture of DMSO and foetal bovine serum. Alternatively a sericin cryopreservative solution consisting of phosphate-buffered saline, 1% sericin, 0.5% maltose, 0.3% proline, 0.3% glutamine and 10% DMSO has recently

been developed and may be better than solutions containing serum that can become infected (Toyosawa *et al*. 2009).

9.2.2 Staining Methods

Differentiation of cell type was difficult in both the fresh and frozen samples. This was highlighted by the fact that neither eosinophils nor mast cells were identified in the analysis of immunostained slides despite their presence being demonstrated by conventional staining methods for cytological examination. Reasons for this failure in staining of eosinophils and mast cells are unclear. The staining process or the stain itself may have disrupted the characteristic appearance of these cell types making it difficult to distinguish these cells from other leukocytes. Previous studies have used morphological characteristics to identify immunostained cells (Wyse *et al.* 2006) and we expected that this method would be adequate in the current study. In experiments performed using respiratory secretions or tissue samples from human and laboratory animals, double immunostaining techniques were commonly utilized to identify macrophages, T lymphocytes, eosinophils and neutrophils (Kitada *et al.* 2001; Lim *et al.* 2000; Maestrelli *et al.* 2001). Confocal fluorescence was utilized in these experiments. We attempted to use double immunostaining without confocal fluorescence but this did not facilitate identification. In previous studies of respiratory samples, immunocytochemical staining of mast cells was not investigated.

Expression of iNOS within mast cells has been demonstrated both by immunohistochemistry and by demonstrating the presence of mRNA by PCR (Gilchrist *et al.* 2002). Mast cells obtained from peritoneal fluid exhibited negligible expression of iNOS compared to peritoneal macrophages (Gilchrist *et al.* 2002). Further study of sections from the respiratory tract of horses diagnosed with respiratory disease would be valuable.

9.2.3 Expression of iNOS and HO-1 in the Equine Respiratory Tract

During planning of the current study it was our intention to document staining in histological sections from different levels of the respiratory tract in horses with IAD, RAO and in control horses. Samples were collected during the study period; however they were

inadvertently destroyed prior to analysis. A previous study of HO-1 immunostaining in airways of healthy horses identified expression of the enzyme in type II alveolar cells, macrophages and neutrophils (Wyse *et al.* 2006). In human airways, HO-1 is distributed widely in the airway submucosa, particularly the airway epithelium and submucosal macrophages (Brunetti *et al.* 2006). In mice, the cytoplasm (particularly in the perinuclear region) of macrophages are the predominant source of HO-1 (Kitada *et al.* 2001).

Expression of iNOS might be expected to be strongest in macrophages (Pechkovsky *et al.* 2002) but may also be seen within the cytosol of a diverse range of cell types. In man, expression of iNOS has been reported in alveolar type II epithelial cells (Warner *et al.* 1995), lung fibroblasts (Romanska *et al.* 2002), airway and vascular smooth muscle cells (Thomae *et al.* 1993; Xue *et al.* 1994; Griffiths *et al.* 1995), bronchial epithelial cells (Adcock *et al.* 1994; Robbins *et al.* 1994; Watkins *et al.* 1997; Pechkovsky *et al.* 2002), mast cells (Gilchrist *et al.* 2002), endothelial cells (Ermert *et al.* 2002), neutrophils (Boucher *et al.* 1999) and chondrocytes (Kobzik *et al.* 1993; Ricciardolo *et al.* 2006). Under healthy conditions iNOS expression may not be detected (Buttery *et al.* 1994).

Bronchial brushings have been collected in anaesthetised children to further investigate the expression of iNOS in respiratory tract epithelium (Lane *et al.* 2004). The brushings were used to extract RNA and cytospin preparations were prepared for immunostaining. All of the epithelial cells stained positive for iNOS whilst there was little expression within macrophages. Bronchial brushing would be a technique that could be adapted for use in horses and might provide a means for further investigation of epithelial expression of iNOS and HO-1.

A chemiluminescent method has been described for measuring NO in equine body fluids (Mirza *et al.* 1999; Hubert *et al.* 2003; Mirza *et al.* 2005) which potentially provides a means of measuring NO in the lower respiratory tract without contamination from the upper respiratory tract. This method also has an advantage over the methods in the current study as it has been shown to be valid on samples exposed to frozen storage for up to a year (Hubert *et al.* 2003). In brief, aliquots are added to a purge chamber of vanadium chloride in 1N HCl under a nitrogen atmosphere. Nitric oxide is liberated into the gaseous headspace and conducted into an NO analyser where it is reacted with ozone to produce a chemiluminescent signal in the 650 to 800nm range. The amount of light is proportional to NO concentration which is calculated from a standard curve of known concentrations (Hubert *et al.* 2003). This method has been used successfully to measure NO

concentrations in equine plasma, abdominal fluid, urine and BALF (Mirza *et al.* 1999; Costa *et al.* 2000a; Hubert *et al.* 2003; Mirza *et al.* 2005). In a study of horses with SPARAO, significant differences in NO concentration were observed in plasma but not in BALF (Costa *et al.* 2000b). Although this finding is contrary to findings in humans with asthma, exhaled NO was measured in the human studies and measurements were potentially contaminated with NO of upper respiratory tract origin (Alving *et al.* 1993; Persson *et al.* 1994; Massaro *et al.* 1995; Kharitonov *et al.* 1996a).

The stage in the disease process at which BALF samples were collected may have influenced the degree of expression of both HO-1 and iNOS. In the face of acute inflammation, iNOS activity is expected to increase whilst HO activity initially decreases with the highest levels of expression being recorded as inflammation is resolving (Willis *et al.* 1996). Willis *et al.* (1996) speculated that during tissue inflammation there may be sequential induction of enzymes with pro-inflammatory effects, for example COX-2 and iNOS, followed by increased expression of enzymes with anti-inflammatory effects, including HO-1, which are responsible for the resolution of inflammatory lesions and aimed at preventing a chronic inflammatory response.

The effects of timing on HO-1 expression in horses could be investigated further; however challenge studies in horses known to suffer from RAO would probably be required to ensure sample collection early in the course of the inflammatory response and to ensure that the stage of the disease process was determined accurately.

9.2.4 Activity of HO-1 in The Equine Respiratory Tract

It was also our intention to investigate the activity of HO-1 in sections of pulmonary tissue from horses with respiratory disease and control animals with neither historical nor clinical evidence of respiratory disease. The activity of HO-1 has been measured in human lung tissue (Tenhunen *et al.* 1968; Carraway *et al.* 1998) and these methods have been employed to determine HO-1 expression in equine lung tissue (Wyse *et al.* 2006). The methods are relatively straightforward and in preliminary work were used in the current study (data not shown). Tissue samples were collected immediately after death from different regions of the lung; cranioventral lobes, caudodorsal lobes and a central location equally spaced between the two other sample sites. The samples were flushed via the larger arteries and bronchioles with 0.9% sodium chloride solution and immediately snap frozen in liquid nitrogen. They were then stored at -80°C for subsequent analysis. Liver was also

harvested as a source of biliverdin reductase and the spleen sampled as a positive control for HO-1 (Carraway *et al.* 1998).

Tissue was homogenized in a buffer of 50mM Tris-HCl, 2mM magnesium chloride and 250mM sucrose (pH7.4) containing the protease inhibitors pepstatin (8.4µM), leupeptin (5.8 µM) and pefabloc (0.2mM) (Sigma). The homogenates were sonicated and then centrifuged for 20 minutes at 18,000 g to separate the cytosolic fraction. The protein content of each cellular fraction was determined by the bicinchoninic acid colorimetric method. Haem oxygenase activity was measured in lung and spleen supernatant by incubation with NADPH, haemin and liver cytosol. Samples were incubated in darkness at 60°C for 1 hour. Bilirubin was extracted in 500µl of chlorophorm and measured as a change in the optical density at 464nm and 530nm using a molar extinction coefficient of 51,819.

This method was used successfully for measurement of HO-1 activity in the lungs of horses subjected to euthanasia at the University of Glasgow for reasons other than respiratory disease and the results were consistent with those of Wyse *et al.* (2006). It was expected that through the study period a small number of cases with respiratory tract inflammation would be subjected to euthanasia even if respiratory tract disease was not the primary reason for euthanasia. Unfortunately, insufficient horses with respiratory disease were recruited to the study of tissue expression of iNOS and HO-1 to generate worthwhile data. Whilst respiratory disease is a common cause of impaired athletic performance in horses and may severely affect a minority of animals at rest it is rarely severe enough to warrant euthanasia. Respiratory disease is commonly encountered by veterinarians in clinical practice and cases are seldom referred for further investigation. Further investigation would be valuable in the future if respiratory tract tissue samples from horses known to be suffering from respiratory disease could be snap frozen in liquid nitrogen immediately following euthanasia.

9.2.5 Measurement of Exhaled Gases

Carbon monoxide and NO may be clinically useful indicators of the activity of HO-1 and iNOS respectively. Measurement of these exhaled gases in addition to measurement of H_2O_2 concentration and pH in breath condensate would have been a logical addition to the present study. Concentrations of exhaled CO have been identified to be associated with the clinical severity of asthma (Zayasu *et al.* 1997; Horvath *et al.* 1998; Yamaya *et al.* 2001) and are reduced in patients receiving corticosteroid therapy (Horvath *et al.* 1998). In a

previous study exhaled CO was measured in 6 horses using a portable electrochemical monitor (Microlyser; Bedfont Scientific) (Wyse *et al.* 2005). Although the monitor was sensitive to 1ppm within a range of 1-500ppm the monitor frequently failed to detect any expired CO (Wyse *et al.* 2005). An increase in expired CO was identified following exposure to hay and straw, however results in both management systems ranged from 0-2ppm (out of 500) indicating the relative imprecision of the instrument (Wyse *et al.* 2005). In human studies similar methods of analysis are used but concentrations of 4-15 ppm are reported (Zayasu *et al.* 1997).

It was our intention to use the same monitor as used by Wyse *et al.* 2005 in order to investigate the relationship between expired CO and HO-1 expression. During initial pilot studies concentrations of CO failed to measure above 1ppm (and in the majority of cases measured 0) in horses diagnosed with clinical respiratory disease. As the methods of measurement available were so imprecise at the required range, expired concentrations of CO were not monitored in the current study as it was considered that the results were likely to have been misleading. If more sensitive and affordable analysers were to become available then they would provide a valuable tool with which to investigate the relationship between expired CO, HO-1 activity and equine respiratory disease. In addition to the imprecision of currently available methods of measurement there are practical difficulties in the assessment of exhaled CO concentrations in animals. Human patients are required to breath-hold for 20 seconds creating an accumulation of CO in the exhaled breath (Zayasu *et al.* 1997). Although this is not possible in horses, a form of re-breathing system with subsequent analysis of collected gas might be a means by which CO could be measured in future. In humans, exercise resulted in a 3-fold increase in expired CO and remained increased for 10 minutes (Horvath *et al.* 2005). Although exercise would not be possible in equine patients with severe respiratory disease, post-exercise testing in athletic horses might provide another means by which exhaled CO monitoring could be improved.

Exhaled CO provides a useful monitor of disease severity in humans and concentrations decrease following treatment with corticosteroids (Horvath *et al.* 2005). If practical methods of monitoring could be employed then this might provide a useful and non-invasive means of assessing respiratory health in horses. Non-invasive monitoring may even be useful in performance horses (in which sedation and airway washes are not possible) and in monitoring the efficacy of inhaled or systemic treatment with corticosteroids. In human medicine, exhaled breath analysis may be used to detect mild inflammation that is not detectable by pulmonary function tests (Choi *et al.* 2006).

Practical considerations prevented the measurement of NO in the current study. A number of different analysers are available, however they all measure NO concentration by chemiluminescence and are extremely expensive (Hanazawa *et al.* 2000). Nitric oxide is one of the earliest and most sensitive indicators of airway inflammation in human asthma and is also produced in other inflammatory diseases such as bronchial hyperreactivity, COPD and interstitial lung disease (Clini *et al.* 1998; de Jongste and Alving 2000). Exhaled NO is a very sensitive indicator of subclinical or early clinical disease and is also sensitive in monitoring responses to inhaled corticosteroids (Choi *et al.* 2006). As NO concentrations are only affected by inflammation and are not influenced by bronchoconstriction, measurement may allow differential assessment of responses to anti-inflammatory medications compared to bronchodilators such as beta$_2$-agonists (Hanazawa *et al.* 2000). Measurement of NO concentration might therefore provide valuable information as to the severity of respiratory disease and responses to treatment in horses in a non-invasive manner.

In addition to being produced in the lower respiratory tract, NO is also derived from the nasal passages, especially in species with paranasal sinuses, and by microbes in saliva (Lundberg *et al.* 1994). Within the lower airways, the conducting airways produce more than the alveolar region (Hanazawa *et al.* 2000). In human medicine, NO has to be collected during mouth breathing. In addition, direct methods of analysis are preferred to offline methods in which exhaled breath is stored (Hanazawa *et al.* 2000). This presents practical difficulties in analysis of breath from horses which cannot be instructed on how to breathe into an analyser. Flow rate has a large influence on the concentration of expired NO and therefore must remain constant (Silkoff *et al.* 1997). In human patients, contribution from the upper airways is controlled by closing the soft palate using balloon catheters, ensuring exhalation against resistance or using a nose-clip (Hanazawa *et al.* 2000). Collection of exhaled breath for measurement of NO is difficult in children less than 7 years of age and not performed in children less than 4. It is therefore likely to prove difficult, if not impossible, to achieve accurate measurements in horses (Choi *et al.* 2006).

The concentration of exhaled NO in exercising horses has been measured (Mills *et al.* 1996a). The authors suggested that the production of NO by the nasal mucosa may not be as significant as previously thought. They found no difference in exhaled NO at high and low flow rates and found no differences when an endotracheal tube was placed during anaesthesia (Mills *et al.* 1996b). The importance of nasal sources may therefore be less

important than the human literature would suggest and may decrease during exercise (Mills *et al.* 1996a). Further investigation into associations between exhaled NO and equine respiratory function are warranted.

9.2.6 Measurement of Exhaled Breath Condensate

Exhaled breath condensate, thought to be vapourised epithelial lining fluid of the lower airways (Chapman *et al.* 2000) has been investigated extensively in human medicine and is practical for use in horses (Deaton *et al.* 2004b; Wyse *et al.* 2005; Duz *et al.* 2009). There is potential for further exploration of the use of analysis of exhaled breath and breath condensate. Although results in equine studies have been disappointing to date (Deaton *et al.* 2004b; Wyse *et al.* 2005; Duz *et al.* 2009) investigation in veterinary medicine lags behind investigation of human respiratory disease, most notably asthma and there are numerous avenues that remain to be explored. In human breath condensate over 300 gases have been identified and several have been associated with respiratory disease (Phillips *et al.* 1999). Pentane, ethane, carbon monoxide and nitric oxide have all been investigated as markers of inflammation and oxidative stress in humans (Kharitonov *et al.* 1994; Horvath *et al.* 1998) and have been identified in horses (Wyse *et al.* 2005). Hydrogen peroxide is the most extensively studied but numerous other products of oxidative stress and inflammation are amenable to measurement by relatively inexpensive ELISA techniques (Wyse *et al.* 2004).

9.3 Wider Significance of These Findings with Respect to Equine Respiratory Disease

The expression of iNOS and HO-1 will be influenced by all inflammatory disease states and may have relevance to respiratory tract diseases that are of importance in horses. Most notably, expression of HO-1 is likely to be integral to the pathogenesis of exercise-induced pulmonary haemorrhage (EIPH); a disease that is of great importance to the equine industry. The importance of HO-1 and its products have not yet been investigated in this disease.

During high-velocity exercise the pressure differential between the capillaries and alveolar lumen increases resulting in rupture of the alveolar capillaries (Hinchcliff 2007). Haemorrhage into the interstitial space and alveoli results in rostral movement of blood in the airways and the presence of blood in the bronchi and trachea (Hinchcliff 2007).

Inflammation of the small airways such as occurs in IAD and RAO is one of a number of potential factors that may increase susceptibility to EIPH (Hinchcliff 2007). Haem released during haemorrhage into the airways may further contribute to the degree of inflammation resulting in a positive feedback loop and a progressive increase in the degree of inflammation within the airways. Haem catalyses the formation of ROS thereby inducing oxidative stress and cell damage (Jeney *et al.* 2002). Haem is highly lipophilic and intercalates readily with the lipid membranes of cells and intracellular organelles, thereby rendering them unstable (Balla *et al.* 1991).

The presence of haem in the airways as a result of haemorrhage is likely to result in induction of HO-1. The significance of haem as a pro-inflammatory mediator is implied by the number of endogenous mechanisms that have been identified to reduce free haem (Wagener *et al.* 2003). In man and laboratory species intracellular proteins such as haem binding protein 23, HO-2 and HO-3 and extracellular proteins such as albumin, haemopexin and HO-1 scavenge free haem (Muller-Eberhard and Fraig 1993; Maines 1997).

Haem oxygenase enzymes perform a vital physiological role in the metabolism of haem during the breakdown of senescent erythrocytes in reticuloendothelial cells and in the turnover of intracellular haemoproteins and cytochromes, thereby ensuring redistribution of haem and iron to the tissues (Maines 1988; Poss and Tonegawa 1997). Haem oxygenase-1 induction by haemoglobin infusion was demonstrated to be protective in a rat model of lipopolysaccharide-induced inflammatory lung injury (Otterbein *et al.* 1995). *In vitro* studies in which HO-1 was produced in excess of physiological requirements provided evidence of conferred protection against haem and haemoglobin-mediated toxicity (Abraham *et al.* 2006). Further investigation of the expression of HO-1 within the lungs of horses with EIPH might therefore be worthwhile and induction of HO-1 might have protective affects in horses affected by the disease.

A therapeutic intervention for EIPH would be of huge potential significance. Almost all thoroughbred racehorses in training have haemosiderophages in BALF indicating a degree of EIPH (McKane *et al.* 1993). Estimates of prevalence based upon endoscopic examination range from 43 to 80% in racing Thoroughbreds (Pascoe *et al.* 1981; Raphel and Soma 1982; Sweeney *et al.* 1990). Whilst prevalence is generally lower in horses performing other athletic disciplines EIPH is also performance-limiting in Quarter horses, polo ponies and eventers (Hinchcliff 2007). The disease is performance-limiting,

recurrence rate is high and the costs to the equine, particularly racing, industry are immense.

As the roles of HO-1 and iNOS become elucidated there may be merit in investigating the effects of corticosteroids on their expression. Corticosteroids are used commonly as a treatment for inflammatory diseases of the lower airways in horses. In asthma, exhaled NO concentrations are reduced by corticosteroid therapy (Hanazawa *et al.* 2000). Numerous murine models have demonstrated the inhibitory effects of steroids on iNOS (Donnelly and Barnes 2002). However, iNOS expression in human airway epithelial cells is steroid insensitive (Nyman *et al.* 1991) and hence inhibition may be indirect via suppression of IL-1β or TNF-α. A glucocorticoid response element is present in the HO-1 promoter and gene expression of HO-1 may be inhibited by corticosteroids *in vitro* (Brunetti *et al.* 2006).

It is likely that the interactions between corticosteroids, HO-1, iNOS and the potentially therapeutic products of these proteins CO and NO will be exceedingly complex. However, as our understanding improves we may identify novel therapeutic targets that will allow improved management of equine respiratory disease.

9.4 Conclusions

Haem oxygenase-1 and iNOS are expressed in equine leukocytes collected from BALF. Convincing evidence that the expression of either HO-1 or iNOS in leukocytes from BALF was increased in horses with evidence of lower respiratory tract inflammation was not obtained, however there was limited evidence of an association between expression of the 2 proteins. There was no evidence of an association between either HO-1 or iNOS expression and markers of oxidative stress measured in EBC. Further investigations into the expression and activity of HO-1 and iNOS ought to be performed with larger numbers of horses at different stages of different respiratory diseases.

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Appendix 1

Clinical Examination Form

The Liphook Equine Hospital

HBLB

UNIVERSITY of GLASGOW

Equine Respiratory Disease Examination Form

(Explanatory Notes Overleaf)

Owner: Horse: Case Number:

Age: Breed: Sex:

Date of Examination:

Nasal Discharge (volume/nature)	
Cough (frequency/nature)	

Medial Nostril Flare (0-4) Θ	
Lateral Nostril Flare (0-4)	
Abdominal Compression (0-4)	
Clinical Score	

Condition Score#		
mucous membs (colour/CRT)		
Heart Rate		
Respiratory Rate		
Rectal Temperature(^{o}c)		

Bronchoalveolar Lavage: Fluid Retrieved (ml)	
Total white cell count (x10^{9}/l)	
Neutrophils (%)	
Macrophages (%)	
Lymphocytes (%)	
Eosinophils (%)	
Mast Cells (%)	

Tracheal Auscultation

Thoracic Auscultation
Auscultation (Rebreathing bag)
Tracheo/ Bronchoscopic findings:

Tracheal mucus score*	

***Tracheal mucus score**:
Grade 0 - None
Grade 1- little, multiple small blobs
Grade 2 – moderate larger blobs
Grade 3 – marked, confluent to stream forming
Grade 4 – Large pool forming
Grade 5 – Extreme, profuse amounts

#Condition Score:
(Carroll and Huntingdon, 1988)

TABLE 1: Body Condition Score System

	Neck	Back and Ribs	Pelvis
0 Very Poor	Marked 'ewe' neck Narrow and slack at base	Skin tight over ribs Spinous processes sharp and easily seen	Angular pelvis – skin tight Deep cavity under tail and either side of croup
1 Poor	'Ewe' neck Narrow and slack at base	Ribs easily visible Skin sunken either side of backbone. Spinous processes well defined	Rump sunken, but skin supple Pelvis and croup well defined Deep depression under tail
2 Moderate	Narrow but firm	Ribs just visible. Backbone well covered Spinous processes felt	Rump flat either side of backbone Croup well defined, some fat Slight cavity under tail
3 Good	No crest (except Stallions) Firm neck	Ribs just covered – easily felt No 'gutter' along back Spinous processes covered, but can be felt	Covered by fat and rounded No 'gutter' Pelvis easily felt
4 Fat	Slight crest Wide and firm	Ribs well covered - need firm pressure to feel 'Gutter' along backbone	'Gutter' to root of tail Pelvis covered by soft fat – felt only with firm pressure
5 Very Fat	Marked crest Very wide and firm Folds of fat	Ribs buried – cannot feel Deep 'gutter' Back broad and flat	Deep 'gutter' to root of tail Skin distended Pelvis buried – cannot feel

Adjust the pelvis score by 0.5 point if it differs by 1 or more points from the back or neck scores to obtain the condition score. (Adapted from Leighton-Hardman 1980.)

ϴClinical Score: ***(Seahorn, 1997)***

$$\frac{\text{Medial Flare} + \text{Lateral Flare}}{2} + \text{Abdominal Lift} = \text{CS}$$

Nostril flare:
0= little or no movement on inspiration
1= slight flare during inspiration, returning to normal as inspiration ends
2= slight flare during inspiration, returning to near normal as inspiration ends
3= greater flare during inspiration and flare does not approach normal position during exhalation
4= nostril remains maximally flared throughout respiratory cycle

Abdominal Lift:
0= no or little movement in the ventral region of the flank
1= slight abdominal flattening with "heave line" beginning to form in the cranial portion of the ventral aspect of the flank
2= obvious abdominal flattening and "heave line" extending to halfway between tuber coxae and the cubital joint
3= obvious abdominal flattening and "heave line" extending beyond halfway between tuber coxae and the cubital joint, but not to the joint
4= obvious flattening and "heave line" extending to the cubital joint

Discharge: nil, scant, moderate, profuse
serous, mucopurulent, purulent, brown/beige, blood tinged

Cough: occasional, frequent single, frequent paroxysmal, at exercise
Productive or non-productive. Dry or wet sounding.

Appendix 2

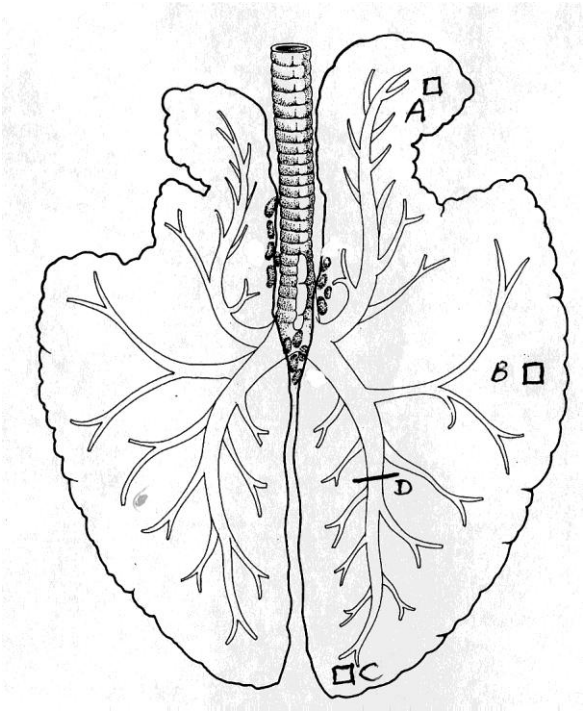
Lung Tissue Collection

Characterising HO-1 and iNOS Expression in the Equine Respiratory Tract

Horses with Recurrent Airway Obstruction euthanased by firearm:

One sample to be taken from each site (A-D) then

1. placed in PBS immediately
2. flushed with PBS using a pipette
3. placed in formalin
4. submitted to Colin for histopathology, HO staining and iNOS staining



Collection from either right or left lung
A-Cranioventral Lung Lobe
B- Cranial end of Caudal Lobe
C- Caudodorsal Lung Lobe
D- Bronchus (of a size that fits comfortably on a microscope slide)

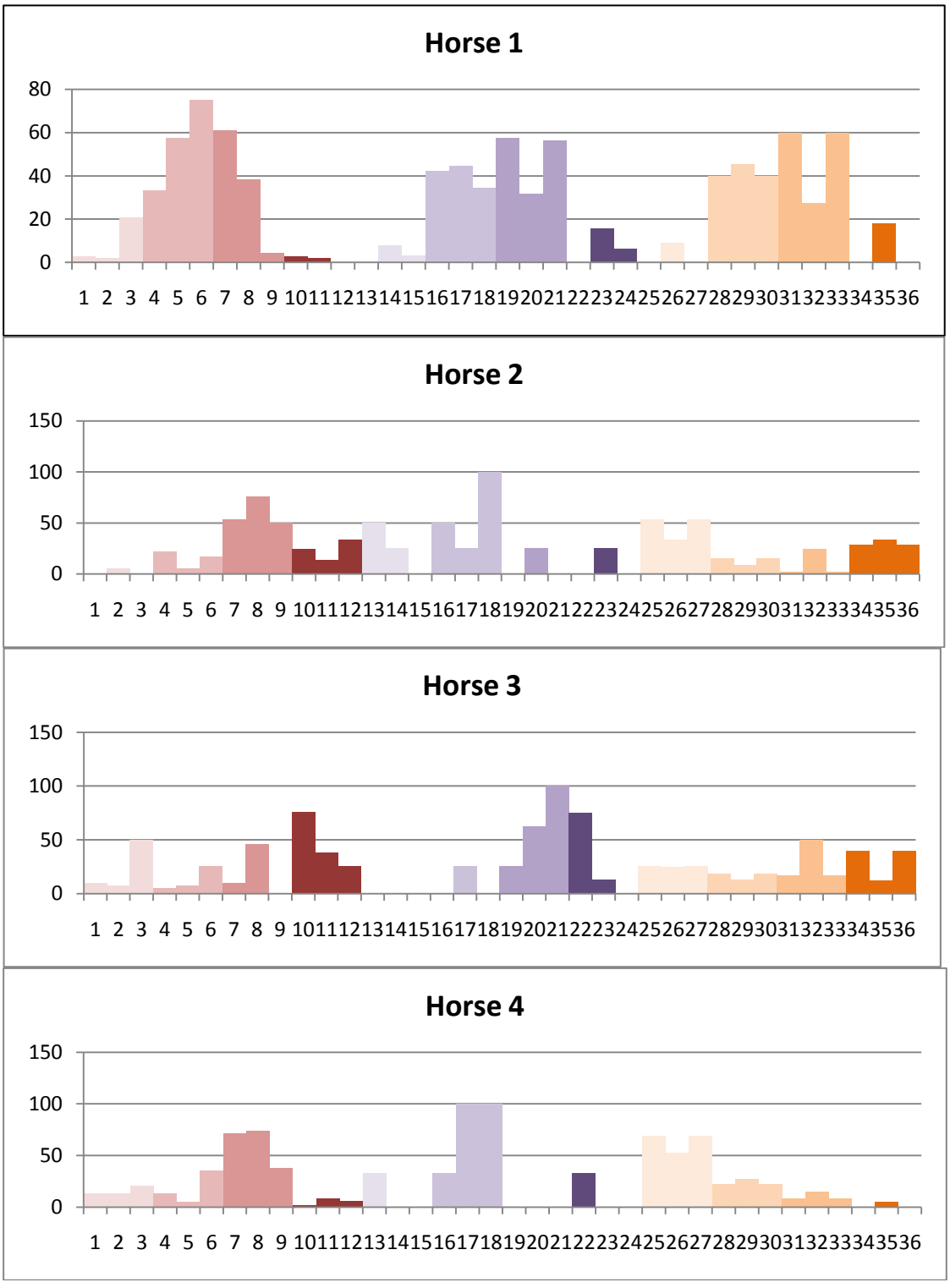
Thank you
Any problems or questions please contact Dave Rendle on 07766080295 or speak to Sandy Love

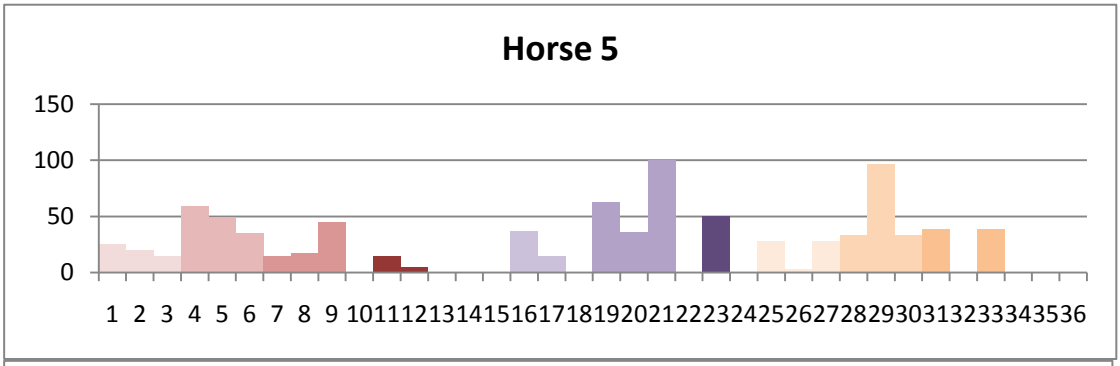
Appendix 3

Frozen Data

Appendix 4

Appendix 4.1. Histograms for each horse showing sequential results of 3 examinations of the same samples stained for HO-1. Results from the 3 sequential examinations are shown consecutively with macrophages grades 0-3 (1-12 on x axis) followed by neutrophils grades 0-3 (13-24 on x axis) then mononuclear cells 0-3 (25-36 on x axis).

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50
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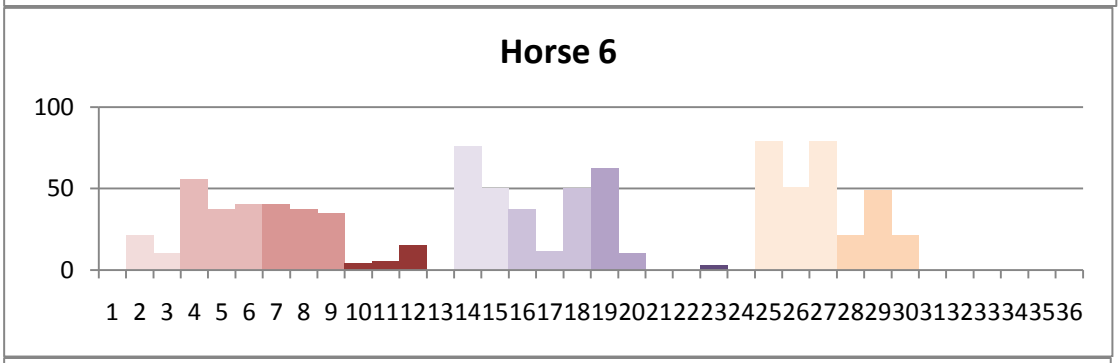
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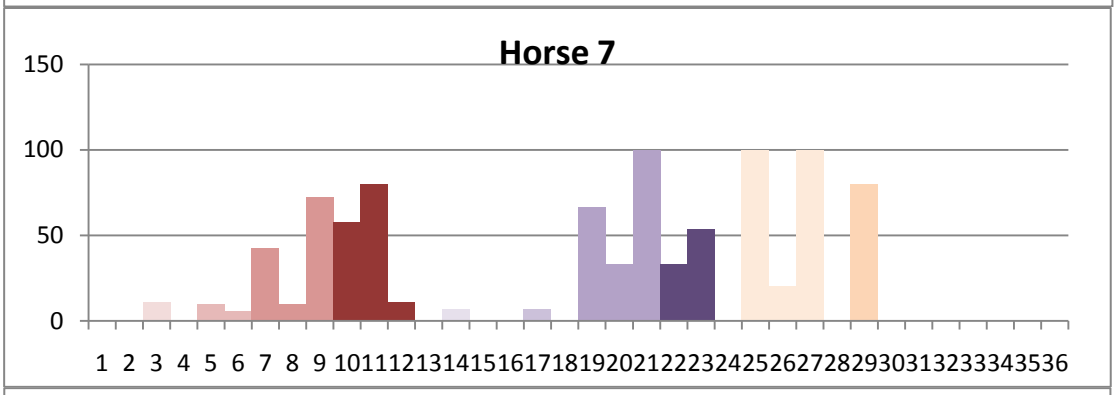
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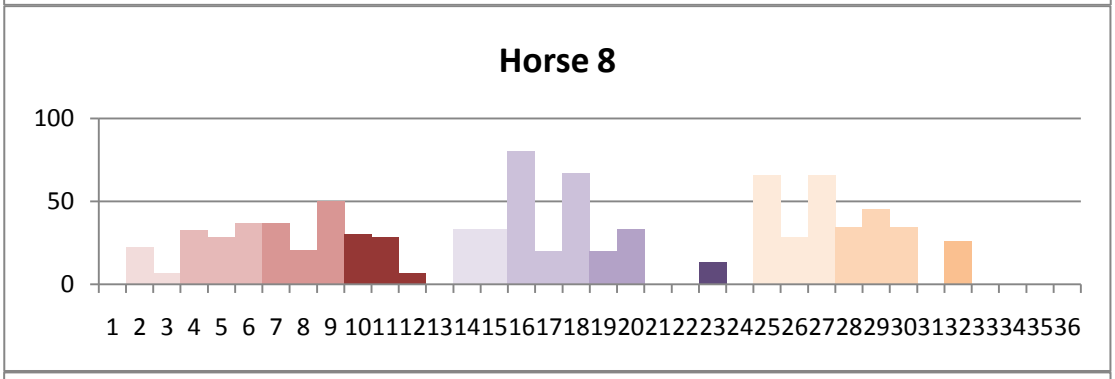
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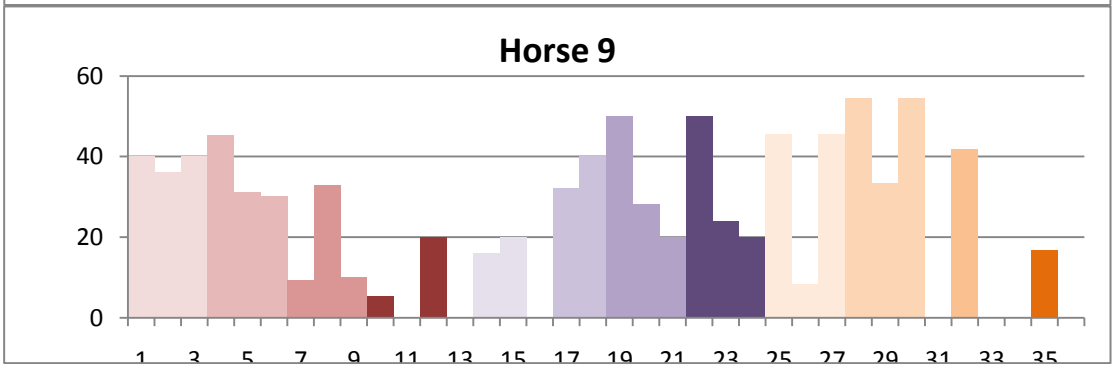
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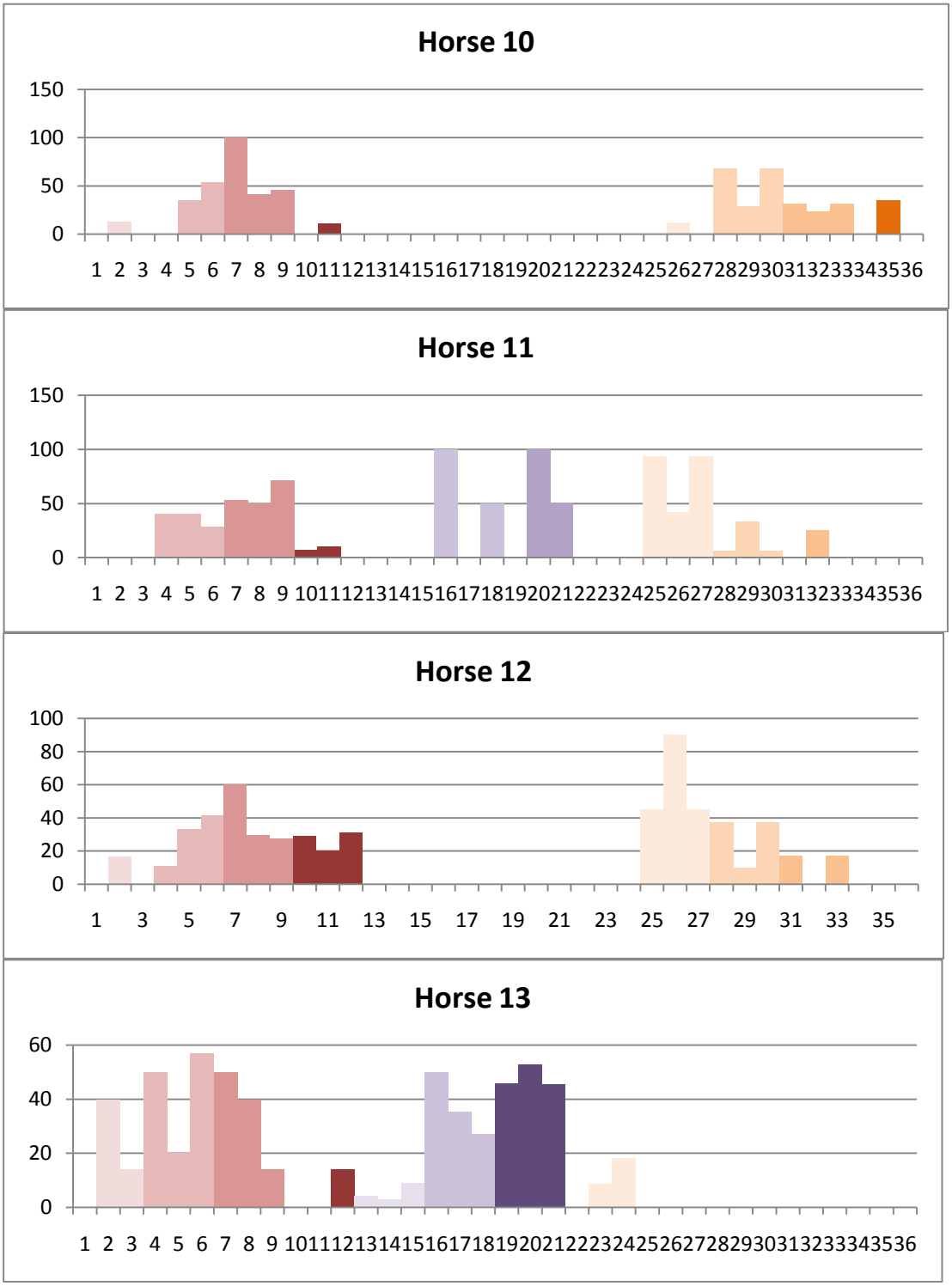
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Horse 11
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100
50
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Horse 12
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1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
Horse 13
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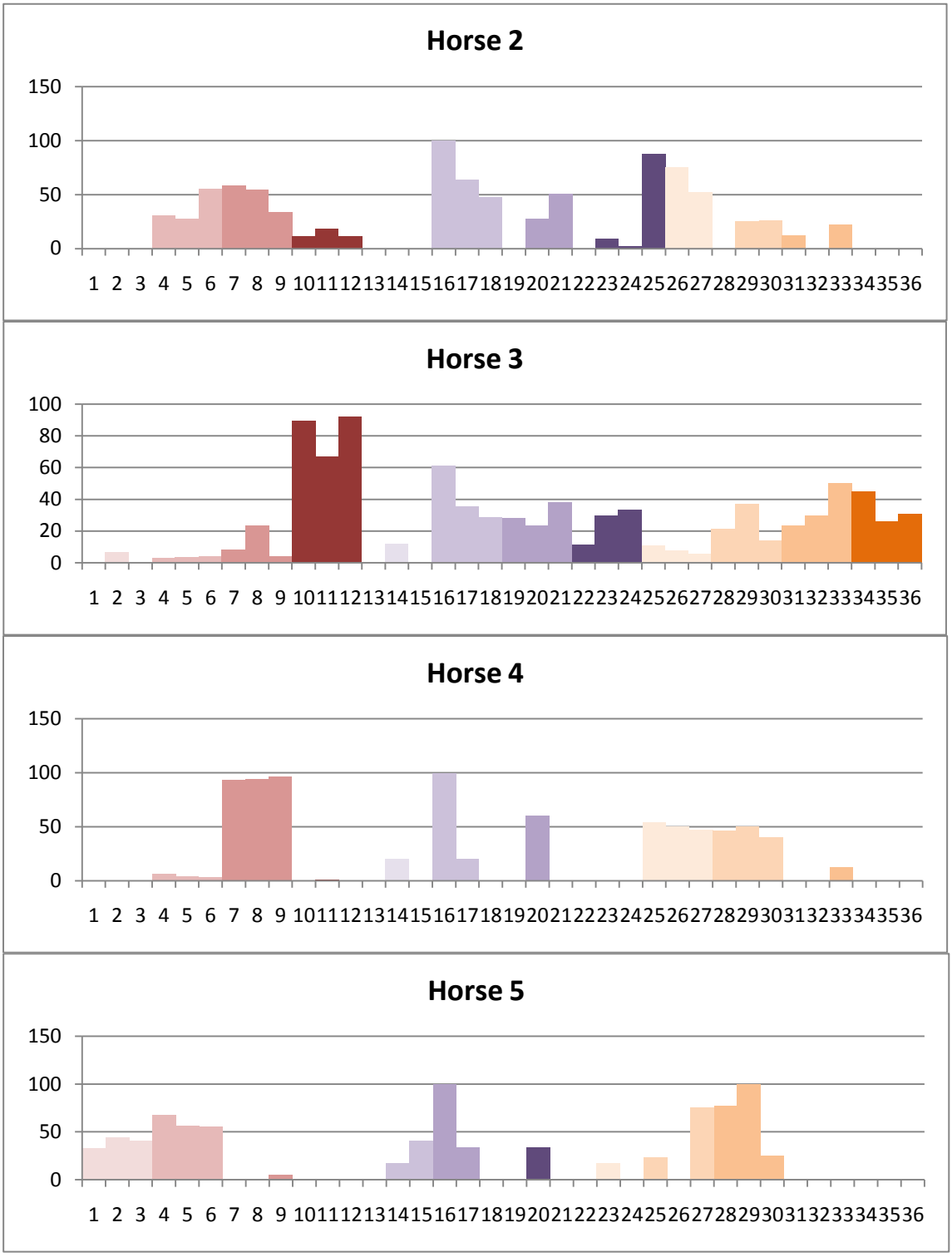
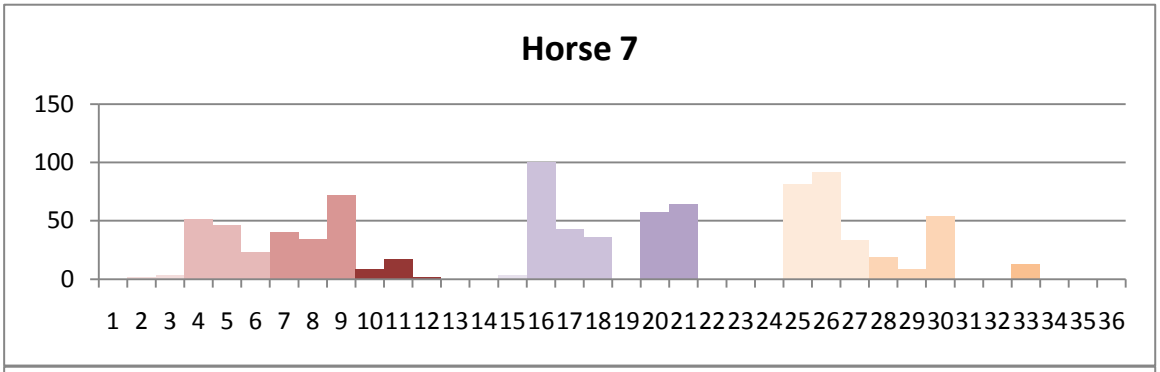
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

Appendix 4.2. Histograms for each horse showing sequential results of 3 examinations of the same samples stained for iNOS. Results from the 3 sequential examinations are shown consecutively with macrophages grades 0-3 (1-12 on x axis) followed by neutrophils grades 0-3 (13-24 on x axis) then mononuclear cells 0-3 (25-36 on x axis).

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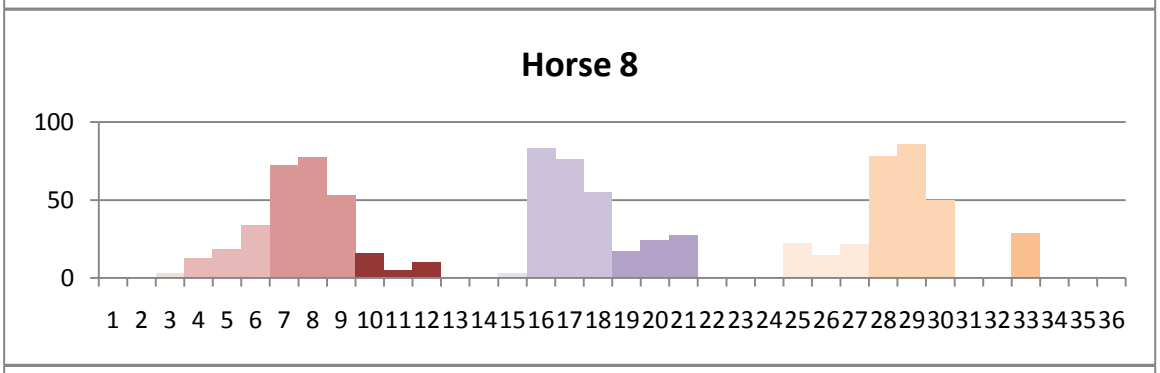
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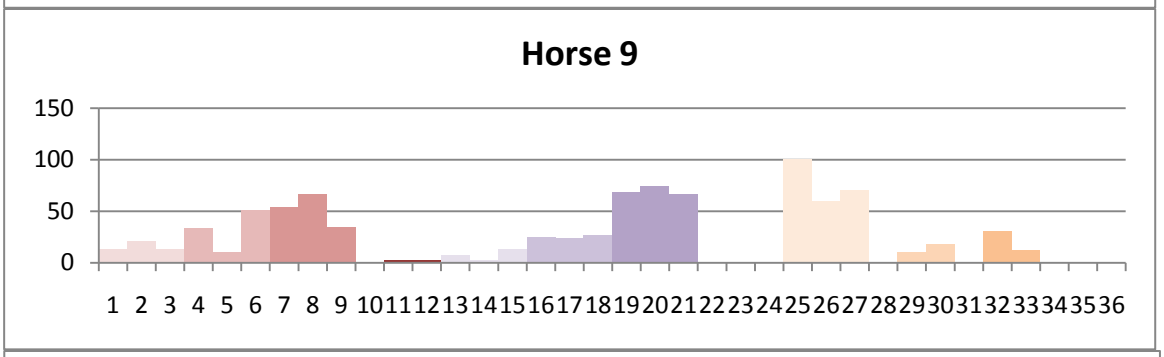
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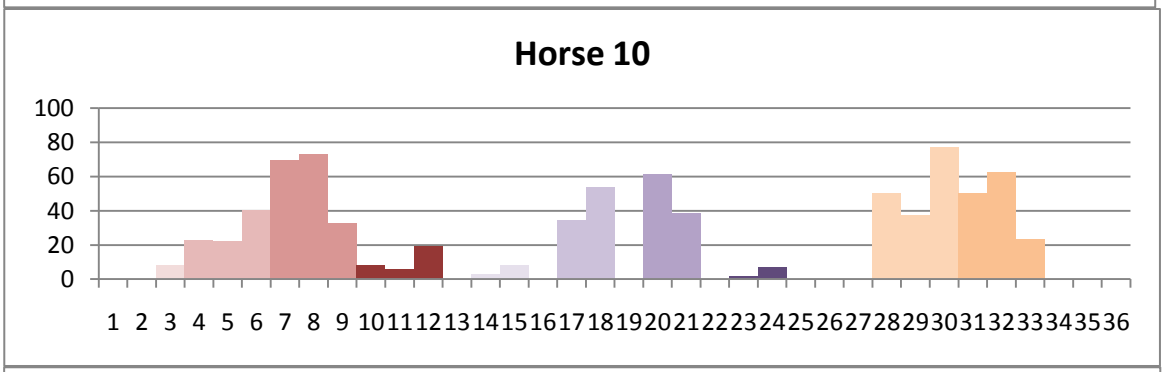
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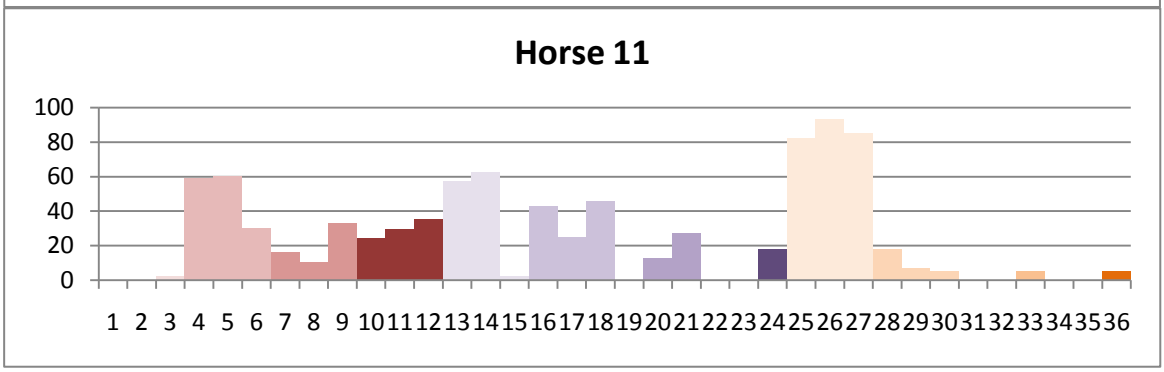
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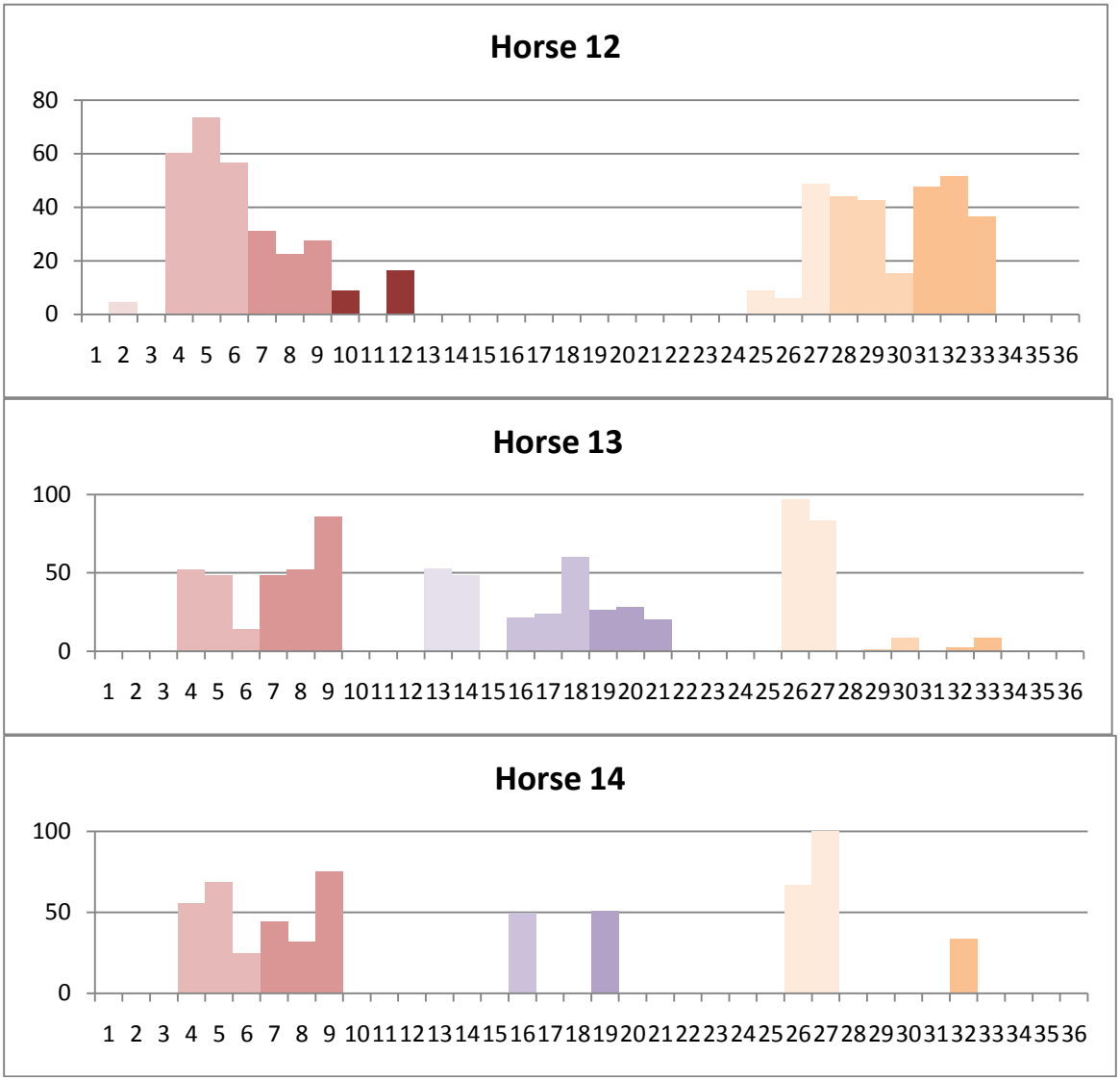
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Horse 13
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Horse 14
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50
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

Appendix 5

List of Manufacturers

Adobe Systems Incorporated, 345 Park Avenue, San Jose, CA 95110-2704, USA

Biomol International, Palatine House, Matford Court, Exeter, EX2 8NL, UK

Bivona Inc, 5700 West 23rd Avenue, Gary, Indiana, 46406USA

BMG Labtech Ltd, PO Box 73, Aylesbury, HP20 2QJ, United Kingdom

Cook Veterinary Products, Cook Medical Inc., PO Box 4195, Bloomington, Indiana 47402-4195, USA

Cranlea and Company, The Sandpits, Acacia Road, Bournville, Birmingham, B30 2AH, UK

Hanna Instruments Ltd., Eden Way, Pages Industrial Park, Leighton Buzzard, Bedfordshire, LU7 4AD, UK

Dako UK Ltd, Cambridge House, St Thomas Place, Ely, Cambridgeshire, CB7 4EX, UK

EV Veterinary Products, The Barn, Wrestlers Farm, Brockhurst Road, Brockhurst, Shropshire, TF11 8NE, UK

Karl Storz, Mittelstrasse 8, Postfach 230, 78503, Tuttlingen, Germany

Microsoft Corporation, One Microsoft Way, Richmond, WA 98052- USA

Minitab Inc, Minitab Headquarters , 3801 Enterprise Drive, State College, PA 16801, USA

Sigma-Aldrich Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK